STUDIES ON THE LOCALIZATION OF TAMM-HORSFALL GLYCOPROTEIN IN THE KIDNEY OF THE SYRIAN HAMSTER BY IMMUNOFLUORESCENCE AND BY LIGHT AND ELECTRON MICROSCOPICAL IMMUNOPEROXIDASE TECHNIQUES, TOGETHER WITH A DISCUSSION OF ITS POSSIBLE ROLE.

A Thesis submitted by
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ABSTRACT

The literature on Tamm-Horsfall (T-H) glycoprotein, which is a regular constituent of the normal urine of man and other mammals, has been reviewed with special reference to its intrarenal localization in the adult Syrian hamster kidney. Its ontogenic development in the hamster was also studied. Localization of T-H protein in the human and rat kidney has been investigated further.

Various methods of fixation and peroxidase labelled antibody techniques were used in addition to immunofluorescent antibody methods. Immuno-electron microscopic techniques for the localization of this glycoprotein have not previously been used and they form an important part of the present investigations. The ultrastructure of the hamster kidney macula densa was also studied in detail. Finally, pilot experiments involving bilateral adrenalectomy were performed in order to study its effect on the T-H glycoprotein in hamster.

The investigations on the distribution of T-H glycoprotein within the kidneys of hamster and human using immunofluorescence techniques have shown that this protein is associated only with the cells of the ascending limb of loop of Henle (ALH) and the distal convoluted tubule (DCT). One striking and probably significant fact which has not been described before is the absence of this glycoprotein in the cells of the macula densa of the hamster and the human kidney.

The next step in the present investigations was to study the localization of T-H glycoprotein in the greater detail provided by immunoelectron microscopy. Using several
different peroxidase-labelled antibody techniques, completely new and interesting observations were made. The results were in accord with those obtained with the fluorescence techniques, but went further showing T-H glycoprotein to be associated with the plasma membrane system of the cells of ALH and DCT but again to be absent in the cells of macula densa, whose membranes were negative.

On the basis of these observations and the interesting fact that T-H appears to be restricted to that part of the nephron believed to possess a low permeability to water, it has been tempting to postulate a possible role for this glycoprotein in the normal mammalian kidney.

Thus: T-H glycoprotein is in some way implicated in the formation of a barrier impermeable to water, but allowing the passage of relatively small molecules such as chloride and associated sodium ions. It is also postulated that the specific absence of T-H glycoprotein from the cells of the macula densa permits them to 'sense' the composition of the luminal fluid without significantly changing its composition. Also, the fluorescent antibody studies made on the kidneys of bilaterally adrenalectomized hamsters suggest a possible involvement of the adrenal cortical hormones in the production and maintenance of T-H glycoprotein.
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MACULA DENSA

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1) To localize the site of production of T-H glycoprotein by the methods of immunofluorescence and light and electron microscopic immunoperoxidase techniques in:
   a) the adult hamster, rat and human kidney, and
   b) the foetal and neonatal hamster kidney.

2) To study in detail the ultrastructure of the macula densa of the distal tubule of the hamster kidney, and

3) On the basis of results obtained by some pilot experiments done on the adrenalectomized hamster and rat kidney, to discuss the possible role of T-H glycoprotein in the physiology of mammalian kidney.
### ABBREVIATIONS

<table>
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<th>Abbreviation</th>
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<tr>
<td>T-H</td>
<td>Tamm and Horsfall glycoprotein</td>
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<tr>
<td>ALH</td>
<td>Ascending limb of loop of Henle</td>
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<tr>
<td>DCT</td>
<td>Distal convoluted tubule</td>
</tr>
<tr>
<td>PCT</td>
<td>Proximal convoluted tubule</td>
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<tr>
<td>CD</td>
<td>Collecting duct</td>
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<tr>
<td>MD</td>
<td>Macula densa</td>
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<tr>
<td>PLP fixative</td>
<td>Periodate-lysine-paraformaldehyde fixative</td>
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<tr>
<td>PAP reagent</td>
<td>Peroxidase anti-peroxidase reagent</td>
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<tr>
<td>PAS Test</td>
<td>Periodic acid Schiff Test</td>
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<tr>
<td>HRPO</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin 'G'</td>
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<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
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<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>PBSA</td>
<td>Phosphate buffered saline 'Dulbecco-A'</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>NaH$_2$PO$_4$</td>
<td>Sodium phosphate (monobasic)</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>Sodium phosphate (dibasic)</td>
</tr>
<tr>
<td>NaCl</td>
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PUBLICATIONS

1) C. L. Foster, R. D. Marshall, K. L. Sikri, F. Bloomfield and D. P. Alexander

The localization of Tamm-Horsfall glycoprotein in the nephrons of the kidney of the Syrian hamster as demonstrated by immunofluorescence and immunoelectron microscopical techniques.


2) D. P. Alexander, C. L. Foster and K. L. Sikri

The effects of bilateral adrenalectomy on the Tamm-Horsfall glycoprotein of the nephron of the kidney of the Syrian hamster - some pilot experiments.


3) C. L. Foster and K. L. Sikri

Some observations on the ultrastructure of the macula densa of the distal convoluted tubule of the kidney of the Syrian hamster.


4) K. L. Sikri, C. L. Foster, F. J. Bloomfield and R. D. Marshall

Localization by immunofluorescence and by light and electron microscopic immunoperoxidase techniques of Tamm-Horsfall glycoprotein in the adult hamster kidney.


The Tamm-Horsfall glycoprotein: Its structure, metabolism and a hypothesis for its role in renal function.


Georg Thieme Publishers, Stuttgart.
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INTRODUCTION
GENERAL REVIEW OF LITERATURE

In 1840, Berzelius noticed a mucinous sediment which was formed when he allowed human urine to stand at room temperature for several hours. Morner (1895) isolated a soluble and an insoluble product which he called 'urinary mucins' from the 'protein-free' human urine and postulated that these mucins originate from the urinary tract. Similarly, Kobayasi (1939) prepared a material which he called 'uromucoid' from the mucinous sediment of urine by precipitation with chloroform. He found that this uromucoid contained 20% reducing sugars, 10% hexosamine and 10% galactose.

Later, Tamm and Horsfall (1950, 52) while working on the haemagglutination caused by influenza and viruses, isolated a high molecular weight glycoprotein \( (7 \times 10^6) \) from the normal human urine which was closely related in properties and chemical composition to the material first isolated by Morner and Kobayasi. They found that this glycoprotein (T-H glycoprotein) has the capacity to inhibit the haemagglutination activity of myxoviruses like some other inhibitory glycoproteins (e.g. those present in submaxillary glands). The presence of this glycoprotein has so far been investigated in only a limited number of species and all the evidence from the work done suggest that this protein is present only in the placental mammals (Wallace and Nairn, 1971), although, Keutal (1965) claimed it to be absent from the urine of racially pure negroes, but this has been denied by others (Schwartz et al., 1973). Cornelius et al. (1963) and Wallace and Nairn (1971) confirmed its presence in the urine of man, horse, sheep, goat, dog, cat,
rabbit, guinea-pig, rat and mouse. Similarly, Pollack and Arbel (1969) found it in monkeys and Dunstan et al. (1974) in the hamster urine.

**Isolation of T-H glycoprotein**

T-H glycoprotein can be prepared in a relatively pure form by applying simple chemical procedures.

Tamm and Horsfall (1950, 52) isolated this glycoprotein from the human urine (pH 6.0 - 7.0) by adding 30 ml of chloroform per litre of urine and then diluting it with an equal volume of distilled water. This diluted urine was brought to 0.58 M NaCl, by the addition of solid sodium chloride and the resulting precipitate washed three times with 0.58 M NaCl solution. Finally, the precipitate was suspended in distilled water and after extensive dialysis it was freeze dried. The yield of this material was approximately 25 mg/litre of urine and contained 19% reducing sugars and 6.1% glucosamine (Tamm and Horsfall, 1952).

Several other methods have been employed for the isolation of fractions from urine which have been found to be closely similar in properties to T-H glycoprotein. Anderson and Maclagan (1955) isolated a mucoprotein from normal human urine by adsorption on benzoic acid. Di Ferrante and Popenoe (1958) isolated a material similar to that of T-H glycoprotein by precipitation with cetyltrimethyl ammonium bromide. Similarly, ethanol (Tamm and Horsfall, 1952) and veronal buffer (King and Boyce, 1959) have also been used for the isolation of closely related proteins.
Uromucoid

According to Boyce and Swanson (1955), the use of 0.58 M NaCl solution for the recovery of virus inhibitory mucoprotein of Tamm and Horsfall (1952) from the urine, generally results in an incomplete precipitation of the mucoprotein. For this reason, they used a substantially higher concentration of NaCl solution (i.e. 1M) for the precipitation of the mucoprotein from human urine, and designated it as Uromucoid. Uromucoid can also be prepared by bringing urine to 0.1 M with respect to veronal buffer at pH 8.6 (King and Boyce, 1959). Similarly, Kirchner and Bichler (1976) isolated a uromucoid fraction from rat urine by gel-electrophoresis on Sephadex G-150 using 0.1 M Tris HCl buffer, pH 8.0.

Uromucoid and T-H glycoprotein are generally considered as two closely identical substances. It is claimed that uromucoid is electrophoretically and ultracentrifugally homogeneous and is serologically (Boyce et al. 1961) and chemically (King et al. 1961) indistinguishable from the T-H glycoprotein. Although, the general pattern of amino acid analysis is the same in both proteins, there are other differences that may be due to the presence of a polypeptide material other than T-H glycoprotein in uromucoid. Since, precipitation with 1M NaCl results in a larger yield than that obtained with 0.58 M NaCl, this would according to Fletcher (1972) seem to be a reasonable possibility. Loosely bound lipids were detected by King et al. (1961) in uromucoid but not T-H glycoprotein which makes them distinguishable. However, work by Fletcher et al. (1970a, b) has shown that
such lipids are also present in the material isolated by precipitation with 0.58 M NaCl. The chemical composition of the two proteins is, in fact, so similar that earlier workers (Keutal et al. 1964; Cornelius et al. 1965; Pollak and Arbel, 1969) used the term uromucoid synonymously with T-H glycoprotein.

Returning now specifically to the hamster T-H glycoprotein, Dunstan et al. (1974) isolated this glycoprotein by precipitation with 1M NaCl from the urine of Syrian hamsters and also from the medium in which baby hamster kidney cells (BHK cells, 21/C13) were growing. This glycoprotein was found to be similar in its chemical and immunological properties to the related proteins from the urine of man and rabbit.

Chemical Composition of T-H glycoprotein

1. Amino Acid Composition

The amino acid composition of T-H glycoprotein was first studied in detail by Maxfield and Stefanye (1962) and later on extended by Friedmann and Johnson (1966b); Fletcher et al. (1970a, b); Marr et al. (1971); Neuberger and Ratcliffe (1972) and Dunstan et al. (1974).

There is a marked similarity between the amino acid composition of T-H glycoprotein from all the species studied so far, although small differences have been found in the relative proportions of the individual amino acids. The amino acid content of rabbit T-H glycoprotein has been found to be 58.6% of the weight of the total glycoprotein as anhydro-amino acid residue (Marr et al. 1971) as compared to 58% in human T-H glycoprotein (Fletcher et al. 1970b). The main differences, however, between the findings of Maxfield
and Stefanye (1962) and Fletcher et al. (1970a, b) are in the values of half cystine, tryptophan and tyrosine. Although, the tyrosine and tryptophan contents of human and rabbit T-H glycoprotein differ, the tyrosine tryptophan ratios are very similar in both species.

2) Carbohydrate composition

It is not usually possible to calculate the exact amount of sugars with great accuracy because of the problems associated with incomplete release of the various sugars and their destruction under the cleavage conditions used (Marshall and Neuberger, 1972). However, by using gas liquid chromatography, Dunstan et al. (1974) found a similar amount of total carbohydrates in the T-H glycoprotein from the urine of hamster, man and rabbit.

The total carbohydrate content of this glycoprotein has been found to be approximately 31% in the rabbit urine (Marr et al. 1971); 28% in the normal human urine (Fletcher et al. 1970b) and 25% in the protein derived from the human urinary casts (Fletcher et al. 1970a). The glucose content, by mass has been speculated as 1.0, 0.9 and 2.9% in the glycoproteins from the urine of man, rabbit and hamster, respectively (Dunstan et al. 1974). Fletcher et al. (1970b) on the other hand, could not demonstrate any glucose at all in the human T-H glycoprotein. Only sugars which are chromatographically identical with mannose, galactose, glucosamine and fucose has so far been detected in the rabbit T-H glycoprotein. Notably, galactosamine is virtually absent from the rabbit T-H glycoprotein while this sugar is present in appreciable amount in the human T-H glycoprotein (Fletcher et al. 1970b).
The value for sialic acid content varies from species to species, ranging from 0 to over 10%. For example, the value for sialic acid in human urinary T-H glycoprotein is in the region of 4% (Fletcher et al. 1970b), whereas T-H glycoprotein isolated from the hamster urine has been found to be devoid of sialic acid (Dunstan et al. 1974).

3) Lipid content

Lipids were found to be absent in the experiments performed on T-H glycoprotein by workers in the early sixties, although, King et al. (1961) reported its presence in uromucoid. However, Fletcher et al. (1970a, b) have shown that small amounts of lipid (about 1%) were associated with the preparations of T-H glycoprotein from normal human and cystic fibrotic patients. Traces of lipid have also been found in the rabbit glycoprotein (Marr et al. 1971). Cholesterol, cholesterol esters, triglycerides, free fatty acids and phospholipids are constant features of the lipid fractions isolated.

In human T-H glycoprotein, the average phospholipid content was found to be 0.26% and the total cholesterol, 0.15% (Fletcher et al. 1970b).

Structure of T-H Glycoprotein

1) Sub-unit structure

There is no agreement among research workers about the sub-unit molecular weight of this glycoprotein. Maxfield and Stefanye (1962) claimed that the ultimate sub-unit of T-H glycoprotein was a polypeptide chain with a molecular weight of approximately 28,000. By treating T-H protein with 50%
acetic acid, Friedman and Johnson (1966a) obtained a molecular weight of 94,000 for each of its polypeptide chains. After oxidising this protein with performic acid followed by ultra-centrifugation, these workers (1966b) produced several different fragments, each with a molecular weight of approximately 32,000. Thus, from the results obtained, they concluded that the sub-units with a molecular weight of 94,000 were actually composed of three or four polypeptide chains linked by disulphide bonds. Similarly, Stevenson and Kent (1970) suggested 100,000 to be the approximate sub-unit molecular weight of a single polypeptide chain. However, Fletcher et al. (1970c) and Marr et al. (1971) by using disc gel electrophoresis in the presence of sodium dodecyl sulphate calculated a minimum molecular weight of 84,000 ± 6,000 in man.

2) Electron microscopic appearance

Studies of Porter and Tamm (1955) have shown that a single T-H glycoprotein macromolecule is an unbranched filamentous structure with a diameter of approximately 100 Å. The length of individual filaments varied between 2,500 Å and 40,000 Å. Maxfield (1961) observed filaments with a diameter between 50 Å to 100 Å and with an average length of 12,000 Å. By negatively staining T-H glycoprotein with silicotungstate at pH 7.0, Bayer (1964), on the other hand, demonstrated that each filament of this glycoprotein was in fact composed of fibrils which possessed a zig-zag appearance with a periodicity of 110 - 140 Å. These observations were later confirmed by Fletcher et al. (1970a) who found the same dimensions in T-H glycoprotein extracted
from urinary casts of humans.

Excretion rate of T-H glycoproteins

According to McKenzie and Patel (1963) the mean excretion rate of T-H glycoprotein in man is 1.94 mg/hour for both normal males and females. They obtained a mean value of 44 mg per day. Boyce et al. (1961) found an excretion rate of 90 mg/day for the uromucoid. Anderson and MacLagan (1955) reported 146 mg/day as the mean excretion value for T-H glycoprotein in man, although, this has been disputed by Boyce et al. (1961) who have demonstrated that their material was contaminated by serum proteins. However, by using radio-immunodiffusion techniques Mazzuchi et al. (1974) found 64 mg to be the average daily excretion rate, which ranged from 1.31 to 5.33 mg/hour. They also found significant differences for each sex, values being 2.98 ± 0.20 for men and 2.32 ± 0.15 mg/hour/1.73 sq. meter of body surface in women.

Site/s of occurrence of T-H glycoprotein in the body

Since its discovery in 1950 by Tamm and Horsfall, there has been little interest shown in this glycoprotein. Although, many authors have performed biochemical studies on this glycoprotein, only a few have actually attempted to discover its localization in the kidney and other organs and tissues.

1) T-H glycoprotein in organs and tissues

The site of origin of T-H urinary glycoprotein in man and other animals has remained a question of controversy. Some research workers have tried to localize its presence in different tissues such as kidney, lung, colon, parotid gland, skin, fat and even striated muscle (Boyce et al. 1961;
Cornelius et al. (1965).

The radioactive tracer work of Cornelius et al. (1965) showed that this glycoprotein is absent in plasma and suggested its de novo formation in the renal tubules. Winzler (1956), Vaerman and Haremans (1959), Grant (1959), McQueen (1962) and Keutal and King (1963) were unable to find substances related to T-H glycoprotein in the plasma and in the water-soluble fractions from leucocytes. Similarly, Grant (1959) could not find any traces of this glycoprotein in seminal, prostatic, urethral or vaginal secretions. Anti-sera to 16 different mucoproteins of human origin all gave negative results when tested against T-H glycoprotein by immuno-diffusion (Schwartz and Pallavicini, 1967). Due to its high molecular weight ($7 \times 10^6$), Stevenson and Kent (1970) suggested that it is highly unlikely for this glycoprotein to be able to pass through the glomerular membranes and concluded the renal tubules to be its actual site of formation. In addition, Edward et al. (1961) found it to be absent from the secretions of isolated urinary-bladder pouches in the dog.

Recently, Tsantoulas et al. (1974) using immuno-fluorescent antibody techniques showed the presence of this glycoprotein in normal human liver cell membranes. Similarly, Masuda et al. (1977) using direct immunofluorescence staining and Ouchterlony double diffusion tests found this glycoprotein to be present in the mucous membrane and muscular layers of the ureter, urinary bladder, urethra and prostate gland from man, rat, cat and dog. Despite the contrary claims made by some workers, the renal origin of
this protein now appears to be well established, since it has been immunochemically identified in the urine produced by the isolated perfused kidneys of dog (Cornelius et al. (1965). This view is strengthened by the immunodiffusion studies of the rat kidney homogenate by Smith et al. (1972) who showed it to contain T-H glycoprotein. Maxfield and Wolin (1962) have shown that this glycoprotein can be recovered undiminished from the urine obtained by urinary bladder or urethral catherization and by cannulating the ureter of a dog at the exit of the renal pelvis. All this evidence suggests that T-H glycoprotein is produced within the kidney itself rather than transported there, although it was postulated by Pape and Maxfield (1964) that this protein may also be produced by the goblet cells of the urinary tract, which in man have been found to occur in urethra and bladder. However, this later suggestion seems improbable, since goblet cells do not constitute a significant feature of the epithelium of the urinary tract.

2) T-H glycoprotein in the kidney

King and Boyce (1963) and McQueen (1962, 66) demonstrated that urinary casts in patients with nephrotic syndrome contained approximately 95% T-H glycoprotein and 5% serum albumin. By using a fluorescent antibody technique, Keutal (1965) demonstrated T-H protein in the matrix of kidney stones of different compositions. These findings were later confirmed by Fletcher et al. (1970b), who have shown that T-H glycoprotein and proteins derived from urinary casts are similar in chemical composition and fibrillar structure. As these casts are usually found in the distal convoluted
tubules (DCT) and collecting ducts (CD), these workers, therefore, assumed that this glycoprotein is probably produced at or above the level of the DCT.

It has been demonstrated by Pape and Maxfield (1964) that human T-H glycoprotein stains positively with periodic acid-schiff (PAS) stain. Because, PAS-positive material is most prominent on the brush border of the proximal convoluted tubules (PCT), it was, therefore, believed that cells of the PCT should be the actual site of T-H glycoprotein formation. Keutal (1965) and Cornelius et al. (1965) claimed that their results using fluorescence antibody techniques confirmed this speculation. Boyce et al. (1961) demonstrated the presence of uromucoid (? T-H glycoprotein) in the kidney in the order of; cortex < medulla < pelvis. Using similar antibody techniques, Hermann (1963) reported it in the cells of CD of the human kidney. Friedman (1966) found some fluorescence with T-H antiserum in the DCT and CD and, probably, loop of Henle but more in the PCT. According to McKenzie and McQueen (1969) the fluorescence found in the PCT was of a non-specific nature and they insisted that the majority of the specific reaction was present only in the cells of the ALH the DCT, especially the macula densa (MD) segment. Their findings were later confirmed by the work of Schenk et al. (1971) on the human, rat, guinea-pig, dog and cat kidney.

Pollak and Arbel (1969) using similar techniques on human biopsy material supported the observations of Friedman and demonstrated the presence of T-H glycoprotein in the loop of Henle, DCT and to some degree in the CD.
They also observed that the distribution of T-H glycoprotein rich casts coincided with those regions of the nephron where the glycoprotein was demonstrated. Similar observations were made by Wallace and Nairn (1971) and Lewis et al., (1972) who reported the presence of T-H glycoprotein in the loop of Henle and the DCT including its MD. These studies were later confirmed by the work of Hoyer et al., (1974). Masuda et al., (1975), on the other hand, although do not specifically mention any particular tubule, claim T-H to be present in all parts of the nephron of the human kidney.

Localization of T-H glycoprotein at the subcellular level

Almost all the work so far on the localization of this glycoprotein has so far been done by immunofluorescence microscopy, which, because of its low resolving power is not suitable for a detailed study at the subcellular level.

Pape and Maxfield (1964) were the first to try to locate the subcellular site of T-H glycoprotein in guinea-pig kidney by immunoelectron microscopy. For their study they used ferritin-labelled rabbit (anti-guinea-pig) T-H antibodies and found the localization of ferritin labelled antibodies along the smooth endoplasmic reticulum and brush border of the PCT cells. From the results obtained, they concluded that T-H glycoprotein is first elaborated in the smooth endoplasmic reticulum of the cells of PCT and then extruded to become associated with the luminal surface of these cells, until it is finally released into tubular urine.

It has been found that the cells of the renal tubules are coated on the luminal surface with a mucous coat which
possesses a fibrillar appearance under the electron microscope and T-H glycoprotein also shows a similar filamentous appearance (Porter and Tamm, 1955; Bayer, 1964). From these observation, Maxfield (1966), Fletcher et al., (1970a) and Fletcher (1972) concluded that this external membrane component is in fact T-H glycoprotein.

By using osmium tetroxide-ruthenium red staining for electron microscopy, Monis et al., (1972) observed membranous glycocalyx-like structures in T-H glycoprotein which resembled the plasma membrane fraction of the transitional epithelium. By using electrophoresis on polyacrylamide gel preparations and staining the gels with amido black B for proteins, sudan black B for lipids and PAS and mucicarmine for carbohydrates, the above authors found similar electrophoretic patterns for homogenates of the transitional epithelium, plasma membrane of this epithelium and T-H glycoprotein, thus suggesting that T-H glycoprotein might originate from the cell surface of the urinary tract.

Similarly, by using differential centrifugation experiments, Grant and Neuberger (1973) found the highest percentage of T-H glycoprotein in the microsomal fraction and postulated that this glycoprotein is synthesized by the microsomes, either throughout the nephron or high up in the cells of the PCT and is then washed down through the tubular lumen into the CD.

Ontogenic development of T-H glycoprotein

Lewis and associates (1972) while studying the ontogenic development of T-H protein, by immunofluorescence methods in the rat kidney, failed to find positive staining in any
part of the foetal kidney and therefore reported that the
development of this glycoprotein is concomitant with the
shift from an intra to an extra-uterine environment. In
other words, they suggested that extra-uterine conditions
are essential for the production of this unusual protein.
Their results were disputed later by Hoyer et al. (1974).

Wallace and Nairn (1971) were the first to show its
presence in the human embryos (from eight weeks of age to
full term) and found this glycoprotein to be located in the
distal convoluted tubules, almost as soon as they could be
recognised, and certainly before they were functioning
fully. Their observations were confirmed by the recent
work of Hoyer and his associates (1974) who have found it
present in both rat and human foetuses.

Ross et al. (1975) obtained samples of amniotic fluid
by transabdominal amniocentesis from pregnant women whose
gestational age ranged between 32 and 40 weeks. After
dialysis and concentration they then tested the concentrate
with the technique of double immunodiffusion in agar against
rabbit serum anti-human T-H and found a reaction of complete
identity between pure T-H glycoprotein from adult normal
urine and amniotic fluid extract. Precipitation lines with
umbilical cord fluid and maternal sera were absent, thus
suggesting a foetal renal origin of this glycoprotein.

T-H glycoprotein and kidney pathology

T-H glycoprotein and the uromucoid (which most authors
consider to be very similar if not identical to T-H
glycoprotein) have been identified by immunological methods
in the matrix of a variety of human kidney stones (Keutal,
Cornelius (1963) found an extensive PAS positive staining throughout the kidney nephrons in the urinary stone disease of sheep, which was in excess of that found in the normal animals. As T-H glycoprotein stains positively with PAS (Pape and Maxfield, 1964), Cornelius (loc. cit) therefore, assumed an extensive production of T-H glycoprotein in the kidneys associated with renal stone disease.

This glycoprotein has also been found to be a major constituent of the hyaline casts present in the kidneys of nephrotic syndrome patients (McQueen, 1962, 1966). It has been shown by Fletcher et al. (1970c) that the protein present in casts possesses a chemical composition and characteristic fibrillar structure similar to that of pure T-H glycoprotein. Maxfield and Wolins (1962), on the other hand, claimed to have discovered a molecular abnormality of T-H protein in patients with cystic fibrosis. However, the work of Friedmann and Johnson (1966a), Stevenson (1969) and Fletcher et al. (1970b) failed to reveal any physicochemical or immunological dissimilarities between the two glycoproteins. Similarly, large amounts of T-H protein have been found in the urine of patients suffering from oliguria or dehydration by Berdon et al. (1969). According to them, this glycoprotein forms a viscid gel thus resulting in tubular blockage during the diuretic phase. Also, the studies of Schwartz et al. (1973) demonstrated that in the instance of acute renal allograft rejection, urinary excretion of this kidney-specific substance increases prior to the clinical diagnosis of rejection (rise in serum creatinine, fall in creatinine
clearance). This increase was consistent with the ongoing process of kidney tubular damage preceding clinical abnormalities which are observed at a point when damage finally results in the deterioration of kidney function.

Similarly, studies of Patel et al. (1964) suggested that T-H glycoprotein aggregation may be a factor in the pathogenesis of acute renal failure by causing tubular obstruction. An experimental tubulo-interstitial nephritis selectively involving the ALH and DCT has been produced in rats by immunization with T-H glycoprotein in complete Freund's adjuvant (Hoyer, 1976). Autoantibodies to this protein have been described in patients with pyelonephritis (Hanson et al. 1976). Recently, T-H deposition in the renal interstitium has been reported (Zager et al. 1978) in medullary cystic disease, chronic pyelonephritis, hydronephrosis and chronic interstitial nephrotic syndrome and by using immunofluorescence microscopy in the glomerular capsular space of patients suffering from hypertension, renal failure and Goodpasture's syndrome (McGiven et al. 1978).

Possible Role of T-H glycoprotein in normal kidney physiology

No firm evidence has so far been produced which might attribute a functional role to T-H glycoprotein which is normally excreted into the urine of all the mammalian species investigated so far. Based on the physical properties of this glycoprotein, Friedmann (1966) implied its usefulness as a lubricating agent and thus helping in preventing damage to the tubular cells. In addition, its
ability to act as a substrate for myxovisus neuraminidase (Tamm and Horsfall, 1950) and its presence in the renal tubular cells led to suggestions that this protein may constitute part of a defence system against viral infections of the urinary tract.

In view of the known property of this protein to bind calcium and other divalent cations, Fletcher (1972) speculated that it may be involved in some way with active transport across the tubular cell membranes. As the cells of the renal tubules possesses a glycocalyx which is believed to be covered with a sialic acid containing glycoprotein coating, it has therefore been suggested that T-H glycoprotein may function to trap and filter materials which are to be reabsorbed (Ericsson and Trump, 1969). The presence of T-H glycoprotein at the luminal border of tubular cells also led to the speculation that this protein is somehow involved in the electrolyte and water transport (Lewis et al. 1972). The protective function of the tubular epithelium and also its involvement in the transport of electrolyte and hydrogen ions is also suggested by Bichler et al. (1976) and Kirchner and Bichler (1976). On the basis of the results obtained, a possible physiological role of T-H glycoprotein in the normal mammalian kidney will be suggested in this thesis.
MACULA DENSA

During the course of investigations on the localization of T-H glycoprotein within the tubular cells of the Golden Syrian hamster kidney, the opportunity was taken to examine the ultrastructure of the macula densa (MD) and the MD region of the nephron. This was done partly because the fine structure of the MD does not seem to have been described in this species before, and partly and more importantly, because of the extreme and constant depletion of T-H glycoprotein in the cells of the MD (see later) which is in striking contrast to the situation of T-H in the ALH and DCT of which the MD is a part. This latter observation, which has also been found to be applicable to the human kidney, could possibly throw some further light on the still problematical role of the MD cells.

There is a relatively small literature on the light and electron microscopical structure of this specialized region of the nephron and this possibly stems from the study of Golgi (1889) who noticed a change in the cells of the distal tubule where it touches the glomerulus and its arterioles. Peter (1907) discovered these cells to be comparatively taller and their nuclei lying closer together than their counterparts in adjacent portion of the distal tubule. Zimmermann (1933) named this accumulation of epithelial cells as the Macula densa and considered it to be an important element of the juxta-glomerular (JG) apparatus. However, as indicated by Cook (1963) there is still a great deal of confusion in the literature concerning
the detailed histology of the JG complex and the nomenclature of its various parts. Some consider the MD to be a part of DCT (Tisher et al. 1968; Latta, 1973; Moffat, 1975) while others a part of the ascending limb (Kaissling et al. 1977). There are even some who consider that MD in fact forms a transition between the DCT and the ALH (Allen and Tisher, 1976).

Ogawa and Sokabe (1971) believe that the JG apparatus (including the MD) and renin are present only in classes higher than Teleosts, while according to Sokabe et al. (1969) extra glomerular mesangium (Barajas, 1970) or 'Polkissen' (Polar cushion) and typical MD are seen only in mammals. This was also found to be true from the observations of Zimmermann (1933), Dunihue and Condon (1940) and Goormaghtigh (1937, 1940). The presence of an MD like structure in the avian kidney has been reported by Edwards (1940), McKelvey (1963) and Berger (1966). Recently, Johnson and Mugaas (1970) and Taylor et al. (1970) also reported an MD like structure in the avian kidney which was similar to the mammalian MD. The claim made by latter authors was based on their light microscopic observations, but Ogawa and Sokabe (1971) using electron microscopic techniques failed to find any similarities between the avian and mammalian MD cells.

One interesting observation about the MD cells of the adult cat and rabbit was first made by McManus (1943, 1945, 1947) who by using silver impregnation techniques and light microscopy, discovered that the Golgi body in these cells is
located on the basal side of the nucleus, an apparent reversal of the position seen in other cells of the nephron. This led him to conclude that under normal conditions some substance from the contents of the tubule passes across the specialized cell of the MD towards the glomerular root. This passage, according to the same author (McManus, 1947), is facilitated by the absence of a basal lamina in the cells of MD, so that these cells are separated from the cells of adjoining arterioles only by their cell membrane. The light microscopical work of Muylder (1945a) confirmed the findings of McManus and later Muylder (1945b) also described two different types of cells, i.e. chromophilic and chromophobic cells ('dark' and 'light' cells) in the MD.

Surprisingly, there is relatively very little literature on the ultra microscopical structure of this specialized region of the nephron which, as suggested by Bucher and Reale (1961a) may be because of the rather laborious and time consuming work required in finding this part under the electron microscope. Finding this region by chance in the usual ultra-thin sections is unlikely and, for this reason, it becomes necessary to carry out a systematic investigation, i.e., to make and examine serially cut 1μm thick sections under the light microscope before cutting ultrathin sections for EM.

Bucher and Zimmermann (1960a) and Bucher and Reale (1961b) while studying the mouse and the rat kidney by electron microscopy, reported the Golgi zone as being infranuclear or at least in the basal third of the cell,
but they did not observe the absence of a basement lamina nor the 'dark' and 'light' cells of Muylder. The basal position of the Golgi system was also confirmed by the work of Thoenes (1961), Hartroft and Newmark (1961), Latta and Maunsbach (1962), Barajas and Latta (1963, 67), Riedel and Bucher (1967) and Latta (1973). Oberling and Hatt (1960a,b), Tisher, et al. (1968) in a study of human renal ultrastructure also noted a continuous basal lamina which along with the so called lacis cells, formed a complicated relationship with the peripheral basement lamina of the glomerulus. They also confirmed the infranuclear position of the Golgi apparatus. In a later series of investigations on rat, mouse and guinea-pig, Bucher and Krastic (1971, 1973, 1975) concluded that there were indeed two kinds of cells - 'dark' and 'light' and that it was in the latter cell type only that the Golgi complex was in an infranuclear position. Finally, Hagege and Richet (1975) confirmed the presence of dark cells in the MD area of the rat kidney although they did not refer specifically to the MD itself.
MATERIALS AND METHODS
MATERIALS

Chemicals

Fluorescein-labelled sheep anti-rabbit immunoglobulin was obtained from Wellcome Research Laboratories (Beckenham, Kent, U.K.). Sheep anti-rabbit globulin serum was purchased from Gibco Biocult (Paisley, Renfrewshire, Scotland, U.K.). Sephadex G-200, Sephadex G-25 and Sepharose 4B were bought from Pharmica (G.B.) Ltd., (London, W.5). DEAE-Cellulose (DE-23) was obtained from Watman Biochemicals (Maidstone, Kent, U.K.). Dextran (Mol. wt. 40,000), Horseradish peroxidase (type VI, RZ-3) were from Sigma (London) Chemical Co. Ltd., (London, S.W.6). Complete Freund's adjuvant was from Calbiochem Ltd., (La Jolla, California, 92037). Nembutal (Sodium-pentobarbital) was from Abbott Lab. Ltd., (Queenborough, Kent, U.K.). Glutaraldehyde was purchased from TAAB Lab., (Reading, U.K.). Polyethylene glycol (PEG 1,000, Mol. wt. 950-1050) and Sodium dodecyl sulphate (SDS) were obtained from BDH Chemicals Ltd., (Poole, Dorset, U.K.). Sheep antirabbit IgG was purchased from Miles Lab. Ltd., (Slough, U.K.).

Animals

Syrian hamsters (*Mesocricetus auratus*) were obtained from Coombehurst Breeding Establishment (Baughwest, Basingstoke, Hants., U.K.). New Zealand white rabbits were from Brock Rabbits Ltd. (Iggulden Farm, Sholden, U.K.).
Isolation of Tamm-Horsfall (T-H) Glycoprotein

1) Hamster T-H glycoprotein

T-H glycoprotein was isolated from the urine of Syrian hamsters by the procedure of Dunstan et al. (1974). Daily urine collections were made from the hamsters which were kept in Jencons metabolic cages. Sodium azide (0.02%) was added to the collected urine and was incorporated throughout the isolation procedure until the final dialysis. The pH of the pooled urine was adjusted to 5 with dilute HCl and the urine dialysed against distilled water at 4°C for at least 24 hours. The urine was then centrifuged at 4°C for 5 minutes (1500g) to remove any food particles and other insoluble material. To the supernatant was added sufficient solid sodium chloride in order to obtain a concentration of 1 M and the preparation left overnight at 4°C. After centrifugation for 20 minutes (1500g, 4°C), the supernatant was discarded and the precipitate dissolved in a volume of water equivalent to one tenth of the original volume of urine. The solution was then dialysed exhaustively against distilled water for at least 24 hours at 4°C. After this, the solution was centrifuged and the small amount of insoluble material obtained, was discarded. The solution was then reprecipitated with 1 M sodium chloride as above, the precipitate dissolved in water, dialysed and finally freeze dried. This fraction was designated 'crude hamster T-H glycoprotein'.

Further purification of this crude glycoprotein was done by gel filtration. For this, the material was
chromatographed on a column of Sepharose 4B (60 cm x 1.6 cm) in buffered sodium phosphate solution containing urea (2.8 mM Na$_2$HPO$_4$; 7.2 mM Na$_2$HPO$_4$; 4 M urea at pH 7.0). Approximately 15-20 mg of crude hamster T-H glycoprotein was dissolved in 1.0 ml of the buffer and applied to the column which was run at 4°C with an upward flow rate of 4.0 ml/hr. Fractions of approximately 3.0 ml were collected and adsorption measurements at 280 nm were made (fig. 1) using a Pye-Unicon SP 700 spectrometer. The purified glycoprotein fractions obtained in the first peak from the Sepharose 4B column, were pooled and dialysed against distilled water. All T-H glycoprotein samples were freeze dried and checked for homogeneity by disc gel electrophoresis. This procedure was carried out by the method of Marshall and Zamecnik (1969) in a Shandon disc gel electrophoresis apparatus (cat. no. SAE 2734; Shandon Scientific Instruments, 65 Pound Lane, London N.W.10) at 4°C. Electrophoretic mobilities were measured relative to bromophenol blue and the gels stained for protein with Coomasie blue. A single band of activity was obtained (fig. 2A) demonstrating that the product was pure.

2) Human T-H glycoprotein

The procedure used for the isolation of human T-H protein was essentially that of Tamm and Horsfall (1952). Urine from normal men was collected in sterile bottles. To this urine, solid sodium chloride was added until the concentration was brought to 0.58 M. On standing at 4°C overnight, sedimentation of the white flocculent precipitate
was obtained, and was separated from the supernatant by low speed centrifugation (1500g, 4°C). This glycoprotein containing precipitate was then washed twice with cold 0.58 M sodium chloride solution. The sediment obtained after centrifugation, was suspended in distilled water against which it was exhaustively dialysed several times at 4°C. After two further precipitations with 0.58 M sodium chloride, the final aqueous solution was centrifuged at 13,000 rpm for 30 minutes. The small amount of insoluble sediment, thus obtained, was discarded and the supernatant freeze dried. Homogeneity of human T-H glycoprotein was checked by disc gel electrophoresis. As in the hamster, the purity of the product was demonstrated by the single band obtained (fig 2B).

Preparation of rabbit (anti-hamster T-H)-IgG

For this procedure, young New Zealand white rabbits were immunised by an intramuscular injection of an emulsion containing 0.5 mg of hamster T-H glycoprotein dissolved in 0.5 ml distilled water and suspended in 0.5 ml complete Freund's adjuvant. Two more such injections were given at fortnightly intervals, each containing 1 mg T-H glycoprotein in water. The rabbits were bled 10 days after the last injection by puncturing the ear vein and the blood was collected in sterile tubes. It was then centrifuged at 3,000 rpm for 15 minutes and the serum collected.

The serum, which now contained anti-T-H antibodies, was then dialysed against Tris-phosphate buffer (0.04 M Tris, 0.005M phosphate buffer, pH 8.6) for at least 24 hours. Pure IgG was obtained by chromatographing the dialysed serum on a DEAE-cellulose column (Sober and Peterson, 1958) which was
equilibrated with the same buffer. Approximately 15 ml of
dialysed serum was loaded at the top of DEAE column and
elution started immediately. About 25 ml of the pooled
fractions containing IgG were collected (fig. 3), dialysed
against three changes of distilled water and freeze dried.
The product was dissolved in the same volume of water as the
serum from which it was derived and the solution stored in
small portions at -20°C. For use in the experiments to be
described, a tenfold dilution of the aforementioned solution
was made with phosphate buffered saline Dulbecco 'A' buffer
(PBSA, Dulbecco and Vogt, 1954).

Preparation of rabbit (anti-human T-H)-IgG

The procedure for the preparation of rabbit (anti-human
T-H)-IgG was similar to that used for the hamster. The
anti-serum obtained after injecting rabbits with T-H
glycoprotein containing emulsion was dialysed against Tris-
phosphate buffer, pH 8.6. IgG fractions from the dialysed
serum were obtained by chromatographing it on a DEAE-
cellulose column and the solution freeze dried. Before use,
the solution was diluted 1:10 with PBSA.

Sheep (anti-rabbit globulin) - IgG

IgG fractions from the commercially obtained sheep
anti-rabbit immunoglobulin anti-serum were isolated by the
method of Sober and Peterson (1958). The procedure was
essentially the same as described for the isolation of IgG
from rabbit anti-T-H anti-serum. For some experiments (the
unlabelled antibody enzyme method of Sternberger and Enzyme
Bridge technique of Mason, see later), the combined fractions
from the DEAE-cellulose column containing IgG (fig.4) were
dialysed against PBSA and the solution diluted two fold
either with PBSA or with Tris buffered saline, pH 7.6
(0.046 M Tris, 0.139 M NaCl). For other experiments
involving HRPO-labelled antibody techniques,(see later), the
IgG fractions obtained from the column were dialysed against
distilled water and the solution freeze dried.

Histochemical identification of the antigen (T-H
glycoprotein) was carried out either by using fluorescein-
labelled antibodies or by HRPO-labelled antibody techniques
(Avrameas and Ternynck, 1971; Nakane and Kawaoi, 1974). The
unlabelled antibody enzyme method of Sternberger et al.
(1970) and the immunoglobulin-enzyme bridge technique of
Mason et al. (1969) were also used.

**Fluorescein-labelled sheep (anti-rabbit) immunoglobulin**

Each millilitre of the commercially obtained fluorescein-
labelled sheep anti-rabbit immunoglobulin, contained about
3 mg antibody protein. The product was dissolved in 1 ml of
PBSA and stored at 4°C. For use in the experiments to be
described it was diluted 8 times with the same buffer.

**Preparations of conjugates of HRPO with sheep (anti-rabbit
globulin) - IgG**

**Method 1**  Coupling of antibody to HRPO with the aid of
glutaraldehyde (the two step procedure of
Avrameas and Ternynck, 1971).

Ten mg HRPO (RZ 3.0) were dissolved in 0.2 ml of
0.1 M phosphate buffer, pH 6.8 containing 1.25% glutaraldehyde.
The solution was allowed to stand for 18 hours at room
temperature and was then filtered through a Sephadex G-25
column (60 x 0.9 cm), equilibrated with 0.15 M sodium chloride. Fractions containing activated peroxidase were pooled and concentrated to 1.0 ml with a Diaflo PM10 membrane. 1.0 ml of 0.15 M sodium chloride solution containing 5 mg purified sheep anti-rabbit IgG, was then added to this solution followed by the addition of 0.1 ml of 1 M carbonate-bicarbonate buffer, pH 9.5. This solution was allowed to stand at 4°C for 24 hours and then mixed with 0.1 ml of 0.2 M lysine solution and the mixture allowed to stand for another two hours at 4°C. This was then exhaustively dialysed at the same temperature against PBSA buffer. The labelled antibodies were then precipitated with an equal volume of saturated neutral ammonium sulphate solution. The precipitates were washed with half saturated ammonium sulphate solution, dissolved in 5 ml distilled water and centrifuged at 20,000 g for 20 minutes. Fractions containing peroxidase-labelled antibodies were then separated from those containing free antibodies by filtration of the preparation on a Sephadex G-200 column (60 x 1.5 cm). Only one asymmetric peak was obtained (fig. 5). The samples containing pure conjugated antibodies (fractions 24-38) were stored in small portions at -20°C.

Method 2  Coupling of antibody to HRPO with the aid of periodate (Nakane and Kawaoi, 1974).

5 mg HRPO (RZ 3.0) were dissolved in 1.0 ml freshly prepared 0.3 M sodium bicarbonate solution, pH 8.1. To this was added 0.1 ml of 1% dinitrofluorobenzene in absolute alcohol and mixed gently for one hour at room temperature. To the above solution 1.0 ml of 0.7 M sodium periodate in distilled water was then added and mixed together until the
colour of the solution turned to greenish-yellow. After this, 1.0 ml of 0.16 M ethylene glycol in distilled water was added and again mixed for one hour at room temperature.

The above mixture was dialysed against several changes of 0.01 M sodium carbonate buffer, pH 9.5 at 4°C and 5 mg sheep-(anti-rabbit globulin) IgG added followed by mixing for three hours at room temperature. To this mixture was added 5 mg sodium borohydride and the solution left overnight at 4°C. After dialysing at 4°C against PBSA, the sample was applied to a Sephadex G-200 column (60 x 1.5 cm) equilibrated with PBSA. Fractions of HRPO-labelled IgG were pooled and their absorbance at 280 and 403 nm were determined. The elution pattern of the conjugate mixture which resulted from the reaction of IgG with HRPO activated with periodate, is shown in fig.6. The first peak (fractions 22-34) contained conjugated IgG, while the unconjugated antibody and HRPO, eluted in the second peak (fractions 43-54). The solution containing conjugated antibodies was divided into small portions and stored at -20°C.

**Method 3 Immunoglobulin-enzyme bridge method**
(Mason et al., 1969)

The antiserum to HRPO was prepared by a series of injections of this enzyme into young rabbits. For the first sensitizing dose, 4 mg purified HRPO (type VI, RZ - 3) were dissolved slowly in 1 ml sterile 0.9% saline and 1 ml complete Freund's adjuvant and the resulting emulsion injected subcutaneously into different sites. Two more such injections were given at fortnightly intervals, each
containing 4 mg HRPO in 0.9% saline. Ten days after the last injection, blood was drawn from the ear vein. The antiserum obtained by centrifugation of the blood at 3,000 rpm was frozen in small aliquots and used undiluted.

**Method**  
**Unlabelled antibody enzyme method**  
(Sternberger et al., 1970)

A soluble peroxidase anti-rabbit peroxidase complex, containing the equivalent of 0.93 mg peroxidase and 2.91 mg antiperoxidase per ml was generously supplied through the courtesy of Dr. Ludwig A. Sternberger (U.S. Army Armament Research and Development Command Chemical System Laboratory, Aberdeen Proving Ground, Maryland 21010). For use in the experiments, it was diluted forty-fold with Tris HCl buffer, pH 7.6 containing saline, together with, 1% normal sheep serum. This solution is referred to as the 'PAP' reagent.

**Number of animals used**

The investigation carried out in this work utilized 85 adult male and female Syrian hamsters, weighing between 110 - 130 gm and this included 15 pregnant females. Of the remaining 70 adult hamsters, 10 were used for light microscopy, 12 for general electron microscopy, 12 for adrenalectomy experiments and those remaining for immunofluorescence and immuno-electron microscopy.

Female hamsters which were certified to be ten days pregnant upon arrival in the laboratory, gave birth regularly on the 16th day postconception. Two pregnant hamsters were sacrificed daily from the 11th up to the 15th day of gestation, and these provided the foetal kidneys used...
in the present studies. Similarly, kidneys were also obtained at one hour and at 1, 2, 4, 8, 10, 13, 15, 18 and 21 days after birth.

To obtain foetal hamsters, the pregnant mothers were killed by decapitation while under ether anaesthesia. After opening the abdominal cavity, each foetus was extracted from the uterus and its kidneys removed after the foetus had been decapitated.

The kidneys from the two human adults were obtained at operation and fixed immediately in the operating theatre.

**Fixation and Processing**

Both immersion and perfusion fixation techniques were used. Immersion fixation was generally used in the cases of foetal and early neonatal hamsters and the adult human kidneys, because, in these cases it was not practicable to use the perfusion method. On the other hand, kidneys from adult hamsters were fixed both by immersion and perfusion techniques.

1) **Fixation and processing for light microscopy**

Fixation for light microscopical studies was done both by immersion and perfusion techniques.

For immunofluorescence and light microscopical immunoperoxidase histochemistry, the kidneys were fixed either in 10% neutral formalcalcium chloride (Baker, 1944), 10% neutral formalin, 2% paraformaldehyde - 0.5% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.4) or in the sodium periodate-lysine-parafomaldehyde (PLP) fixative of Mclean and Nakane (1974) (for the detailed preparation of this fixative see p.59).
In addition, for fluorescence microscopy, some hamster kidneys were also fixed by immersion in acetone and 80% ethanol.

The foetal hamster kidneys were fixed whole, the neonatal kidneys cut in halves and the adult hamster and human kidneys, cut into small pieces, before fixation. In the cases of immersion fixation, specimens were generally left in the fixative overnight at room temperature.

After fixation, the tissues fixed in formal calcium chloride or neutral formalin were rinsed for one hour in running tap water to remove the excess fixative before proceeding with dehydration. Similarly, specimens fixed in PLP fixative were washed thoroughly with sodium phosphate buffer. After dehydration in graded alcohols and clearing in chloroform overnight, the specimens were embedded in 56°C paraffin-wax. Kidneys, fixed in acetone and 80% ethanol, were rinsed in absolute ethanol, before embedding in paraffin-wax. 5 μm serial sections were cut on a Spencer microtome.

2) Cryostat sectioning for fluorescence microscopy

Hamsters were killed by decapitation while under anaesthesia and the kidneys dissected free of the surrounding tissue. Because of the large size, kidneys were bisected and each half placed cut surface down on to the chuck. A few drops of saline were applied sparingly around the block to aid its sealing to the chuck, followed by quick quenching in the liquid nitrogen. Cryostat sections were cut at 8 - 10 μm. They were then transferred to a cold slide, dried at room temperature and used unfixed for fluorescence microscopy.
3) **Routine electron microscopy**

The general ultrastructure of the normal and adrenalactomized hamster kidney was studied. For this, both immersion and perfusion fixed (see later) kidneys were used, although, perfusion fixed material gave better results.

(a) **Composition of the fixatives used for routine electron microscopy**

Several different fixatives were tried for the fixation of kidneys used for routine electron microscopy. Different strengths of glutaraldehyde used in the fixatives were as follows: -

1) 6% Glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3.

2) 6% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4.

3) 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.3.

4) 3% glutaraldehyde and 3% dextran in 0.1 M sodium cacodylate buffer at pH 7.4.

5) Karnovsky's full strength glutaraldehyde (6%) in 0.2 M sodium cacodylate buffer, pH 7.3 (with or without dextran).

6) Half strength Karnovsky's glutaraldehyde (3%) in 0.2M sodium cacodylate buffer, pH 7.3 (with or without dextran).

All solutions were freshly prepared and used either at 4°C, room temperature or at 37°C. Of the six fixatives mentioned previously, number 6 proved to be the one of choice.
b). Fixation by dripping and immersion

Fixation by dripping the fixative on the surface of the kidney \textit{in vivo} with an intact vascular supply was done by pouring glutaraldehyde fixative on the kidney surface. After fixation for approximately 15 minutes \textit{in situ}, the kidneys were removed, cut into smaller pieces and reimmersed in the same fixative for an additional two to three hours.

c). Fixation by vascular perfusion

For vascular perfusion, the modified apparatus of Rossi (1975) was used. It consisted of three glass bottles, each with a capacity of approximately 500 ml, with airtight rubber corks. The corks were pierced by two 3 - 4 mm holes through which lengths of glass tubing were inserted. The first bottle contained water and served as a pressure bottle. One end of this bottle was connected to the manometer and the other through a Y shaped joint, was connected on one side to a rubber bulb syringe and on the other side to the short ends of the other two bottles with the aid of another Y-shaped joint (Fig. 7). Similarly, the long ends of the second and third bottles were connected through a three-way valve to the syringe needle used for insertion into the abdominal aorta or the heart. The second bottle contained fixative, while the third bottle contained buffer solution (either sodium phosphate or cacodylate depending upon the buffer used in the fixative) for rinsing. In order to prevent blood clotting during perfusion, 500 units of heparin were added to each 100 ml of rinsing fluid.

After making the system airtight, the pressure was raised
slightly above the normal blood pressure level for the animal (hamster, 130 - 140 mg mercury) and freed of air bubbles by manipulating the three-way valve.

The animals were anaesthetized with an intraperitoneal injection of 0.2 ml of sodium pentobarbital (Nembutal). They were then fixed by vascular perfusion of the kidneys either retrograde through the abdominal aorta as described by Maunsback (1966a) or via the heart (Pease, 1964; Bodian and Taylor, 1963).

**Method 1**

When the animal gave no reflex response, it was pinned out on a cork board, the abdominal cavity opened and the viscera displaced until the dorsal aorta and the inferior vena cava became visible. The aortic bifurcation was freed from the attached muscles, and the aorta was then clamped with a pair of round edged surgical forceps just distal to the renal arteries. A 21 gauge syringe needle was inserted heartwards into the aorta about 1 cm from its distal bifurcation. After making a wide cut in the vena cava, the perfusion was started immediately. During the first three to four minutes the kidneys were rinsed by admitting the buffer solution into the aorta through the three way valve. As soon as the kidneys started to pale, the perfusion was switched from buffer to the fixative, taking care not to allow the perfusion pressure to fall. A small amount of fast green was added to the fixative in order to create a built-in marker by which the effectiveness of the perfusion could be judged.
Method 2

After nembutal anaesthesia, the thoracic cavity of the animal was opened and after grasping the heart with a pair of blunt forceps, the syringe needle was inserted into the left ventricle close to the apex. The perfusion was started immediately after opening the right atrium. Kidneys were first rinsed with the buffer solution before switching to the fixative.

The perfusion of the fixative was usually continued for a minimum of 10 minutes, after which the kidneys were removed. Specimens were then excised and cut into smaller pieces from different levels of the cortex and medulla, and immersed in the same fixative for another two to four hours.

Of the two perfusion methods tried, the best results were obtained with the first.

Post fixation, dehydration and embedding.

After fixation, the specimens were rinsed in the same buffer as was used during perfusion and post-fixed in Palade's osmium tetroxide (Palade, 1952) solution for one hour at 4°C. After washing in distilled water, specimens were dehydrated in graded alcohols, embedded in TAAB resin (medium hardness) and left overnight in a 60°C oven for polymerization.

Sections were cut on a Cambridge-Huxley ultramicrotome (Cambridge Instruments Ltd.) with glass knives and mounted on uncoated 200 mesh copper grids. Sections were stained first, for 3 minutes at room temperature either in 3% aqueous (Watson, 1958b) or saturated uranyl acetate in methanol
(Stempak and Ward, 1964) and then for another three minutes in modified Reynold's lead citrate (Venable and Coggeshall, 1965). The grids were examined on either a Miles MR 60C or AEI-EM 6B electron-microscope at 60 kV.

**Acid Phosphatase (Localization of lysosomes)**

Acid phosphatase activity in the cells of the macula densa of the adult hamster kidney was demonstrated by Barka and Anderson's modification of Gomori's method. (Barka and Anderson, 1962). For this, 30 µm thick cryostat sections from the perfused-fixed material were used. Fixation was carried out in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4. After washing in 0.1 M tris-maleate buffer pH 5.2, sections were transferred to the incubating medium and left for 40 minutes at 37°C. The medium was prepared by mixing 10 ml of 0.1 M tris-maleate buffer (pH 5.2), 10 ml distilled water, 10 ml of 1.25% sodium B-glycerophosphate and 20 ml of 0.2% lead nitrate. Following incubation, specimens were rinsed in 0.2 M cacocylate buffer and post-fixed and embedded for electron microscopy, in the normal manner. Substrate free controls were also prepared.

Erlich's haematoxylin and 1% alcoholic eosin were used for general purpose studies, while for the differentiation of neutral and acid mucopolysaccharides, hamster kidney sections were stained with Nowry's alcian blue/PAS technique (Pearse, 1969). For the localization of Golgi bodies, both Aoyama's silver impregnation technique (Baker, 1950) and the osmium tetroxide technique of Kolatschev (Baker, loc. cit.) were used.
Immuno-histochemical techniques for the localization of T-H glycoprotein in the hamster and human kidneys.

1. **Immunofluorescence Histochemistry** (indirect 'sandwich' method)

   Tissue sections were stained by the indirect (sandwich) immunofluorescent method, using rabbit (anti-hamster or human T-H) immunoglobulin 'G' as the first layer and fluorescein-labelled sheep anti-rabbit globulin as the second layer. Control tests were carried out by using labelled sheep antibody alone.

   For fluorescence microscopy, the dewaxed and hydrated kidney sections were first soaked in PBSA, pH 7.2 for 3 - 5 minutes. Sections were then placed for 10 - 15 minutes in rabbit (anti-hamster or human T-H) IgG at room temperature, washed for four to five minutes in buffered saline and reimmersed in fluorescein-labelled sheep anti-rabbit globulin for another 10 - 15 minutes at room temperature. After rinsing the sections thoroughly with PBSA, they were then mounted under sealed cover slips in the same buffer. Occasionally, buffered glycerol and elvanol (Courser et al. 1974) were also used as mounting medium. Tests were also made on untreated fixed or unfixed cryostat as well as fixed paraffin embedded sections for intrinsic fluorescence.

   The preparations were examined under a Leitz microscope, fitted with a Leitz dark-ground condenser and a quartz/iodine light source, provided with a Turner filter (Gillet and Seibert FITC 3). A barrier filter (Wratten B15) was incorporated into the microscope. Alternatively, a Gillet and Seibert conference microscope, fitted with a Zeiss IV FL
epifluorescence condenser with an HBO 50w mercury vapour lamp was also used. Filter sets for this microscope consisted of FITC selective observation filter with blue excitation at 450 - 490 nm.

The illumination, time exposure and photographic processing were standardised throughout. Photomicrographs were taken at 40 seconds exposures on black and white Ilford HP4 and HP5 films.

2. Immunoperoxidase Histochemistry

a) At the light microscopical level

Light microscopical immunoperoxidase histochemistry was performed on the hamster and human kidneys by the modified method of Burn et al., (1974) and Pich et al., (1976). The kidneys were fixed either by immersion or by perfusion and 5 μm paraffin sections were cut, as previously described.

In a preliminary study, peroxidase labelled sheep anti-rabbit IgG, prepared either according to the method described by Avrameas and Ternynck (1971) or Nakane and Kawaoi (1974) was used. Alternatively, the soluble peroxidase-anti-peroxidase complex of Sternberger et al., (1970) was also applied. The last method, however, gave better results and was employed throughout for light microscopy.

The dewaxed sections were first treated for 20 minutes at 4°C with cold 1% hydrogen peroxide (H₂O₂) in methanol (9.5 ml 6% H₂O₂ + 40.5 ml methanol) to block the endogenous peroxidase activity (Streefkerk, 1972). The sections were then washed in PBSA for 15 minutes and a) covered with one drop of undiluted normal sheep serum
for 10 - 15 minutes in order to minimise non-specific background staining

b) washed in PBSA (10 minutes)

c) treated with rabbit anti-hamster or human T-H IgG (diluted 1:10 with PBSA) at room temperature for 30 minutes.

d) washed with several changes of PBSA (30 minutes)

e) treated with sheep anti-rabbit globulin IgG (undiluted) at room temperature for 30 minutes

f) washed with several changes of PBSA (30 minutes)

g) treated with few drops of soluble peroxidase-anti-peroxidase complex (diluted 1:40 with Tris buffered saline).

and finally, after thorough washing in PBSA (30 minutes to one hour) sections were histochemically stained for peroxidase using diaminobenzidine and H₂O₂ (Graham and Karnovsky, 1966) for 10 - 20 minutes. For this, the sections were placed at room temperature in a freshly prepared solution of 3,3' diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6 containing H₂O₂ (0.005% - 0.03%). The saturated solution was prepared by dissolving varying amounts (3 - 10 mg) of diaminobenzidine in 10 ml of buffer and filtering. However, after a series of experiments, diaminobenzidine at a concentration of 7 mg/10 ml buffer was found to be most suitable for the present investigations and was used at this concentration throughout.

After washing in distilled water (5 minutes) the sections were brought through graded alcohols to xylene and mounted in XAM. In some experiments, following the
enzymatic reaction, the sections were washed with PBSA for 10 minutes, rinsed briefly in distilled water and osmicated for 5-10 minutes with a few drops of a 2% aqueous solution of osmium tetroxide. The osmication darkened the brown reaction product and prevented its fading or diffusion, which otherwise occurred after several months of storage. Alternatively, sections were counter-stained with Erlich's haematoxylin for the precise identification of the reacting tubules.

Control tests were performed either by substituting anti-T-H antibodies with normal rabbit serum or by omitting step c altogether from the above stages.

b) At the ultrastructural level (immunoelectron microscopy)

Several different techniques of fixation, tissue processing and protein-coupling were employed to demonstrate the intracellular localization of T-H glycoprotein in the foetal, neonatal and adult hamster kidneys and also in the adult human kidney.

b.1) Fixatives

Paraformaldehyde and purified glutaraldehyde were used at varying concentrations. The concentrations of various fixatives were as follows:

  i) 4% Paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4
  ii) 2% Paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4
  iii) 1% Paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4
  iv) 2% Paraformaldehyde and 1.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4
v) 2% Paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4
vi) 1% Paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4
vii) The sodium periodate-lysine-paraformaldehyde (PLP) fixative of McLean and Nakane (1974), prepared as follows was also used:

0.1 M dibasic sodium phosphate was added to 0.2M lysine-HCl in distilled water until the pH of the solution was 7.4. This was then diluted to 0.1 M lysine with 0.1 M sodium phosphate buffer, pH 7.4. Just before use, 3 parts of lysine-phosphate buffer were mixed with 1 part paraformaldehyde solution and solid sodium m-periodate was added. The paraformaldehyde concentration was varied from 1 to 2% and that of periodate from 0.5 to 0.1 M.

b.2) **Fixation of tissues**

As in the case of routine electron microscopy, both perfusion and immersion fixation were applied. In general, adult hamster kidneys were fixed by perfusion while foetal and neonatal hamster kidneys, and human biopsy specimens were fixed by immersion at room temperature for 4 - 6 hours. The tissues were subsequently washed with several changes of sodium cacodylate or phosphate buffer, containing 7% sucrose.

**Preparation of the tissue for immunoelectron microscopy**

A) **Use of frozen sections** (Kuhlmann and Miller, 1971)

Prior to the treatment with antibodies, frozen sections
(10 - 30 μm thick) were cut from the small blocks of fixed tissue. For this purpose, slices were first incubated in 10% dimethyl sulfoxide (Kuhlmann and Miller, 1971) buffered with 0.2 M sodium cacodylate, pH 7.2 or 0.05 M sodium phosphate buffer, pH 7.2 (depending on the buffer used during fixation), for one hour at room temperature. Slices were then snap frozen in liquid nitrogen and sections cut on the freezing microtome (Avrameas and Bouteille, 1968; Kuhlmann and Miller, 1971). The sections were collected and washed in sodium cacodylate, sodium phosphate or PBSA for approximately 30 minutes and then procedure I, II, III or IV was applied.

**Procedure I**: was based on that of Avrameas and Ternynck (1969). The sections were immersed for periods of 6 to 18 hours at 4°C in rabbit-(anti hamster or human T-H glycoprotein)-IgG and then washed for 2 - 4 hours in several changes of PBSA buffer with constant stirring. They were immersed for 6 - 18 hours at 4°C in the PBSA diluted solution of the HRPO conjugated sheep-(anti rabbit globulin)-IgG prepared with the aid of glutaraldehyde. Sections were then washed as before with PBSA before being histochemically stained for peroxidase with 3,3'-diamino-benzidine and H₂O₂ (Graham and Karnovsky, 1966) for 20 - 30 minutes.

**Procedure II**: was based on that of Nakane and Kawaoi (1974). The slices were treated as above with rabbit-(anti hamster or human T-H glycoprotein)-IgG and washed with PBSA buffer before immersion in the PBSA diluted solution of sheep-(anti-rabbit globulin)-IgG-HRPO conjugate formed with
the use of sodium periodate and potassium borohydride. The slices were then treated as in procedure I.

Procedure III: was based on that of Immunoglobulin-enzyme bridge technique of Mason et al., (1969). The cryostat sections were treated with the following, in order:

a) rabbit (anti-hamster or human T-H)-IgG  
b) sheep (anti-rabbit globulin)-IgG  
c) rabbit anti-peroxidase serum (undiluted)  
d) MRPO solution

The rehydrated sections were incubated for up to 18 hours with each reagent at 4°C. Between each stage, the sections were washed with several changes of PBSA (2 - 4 hours).

In order to introduce the enzyme label into the bridge after anti-peroxidase serum had been applied, the sections were incubated in a 1.5 mg/100 ml solution of peroxidase (type VI) in PBSA.

Antigen (T-H glycoprotein) was visualized by using diaminobenzidine and \( \text{H}_2\text{O}_2 \) treatment.

N.B. The final reaction product obtained after applying this technique, was not strong enough to justify its routine use and was, therefore, abandoned.

Procedure IV: This was based on the method described by Sternberger et al., (1970). Cryostat cut tissue sections were immersed for 2 hours at 4°C in a 1 in 10 (v/v) dilution of the normal sheep serum in Tris-HCl buffer, pH 7.6 (0.046 M in Tris) containing 0.139 M NaCl and then in rabbit-
(anti-hamster or human T-H glycoprotein)-IgG solution for 18 hours at 4°C. They were then washed with several changes of Tris buffered saline followed by treatment for another 18 hours at 4°C with a solution in Tris-NaCl buffer containing 1% normal sheep serum of sheep-(anti-rabbit globulin)-IgG. After washing with several changes of the Tris-NaCl buffer, the slices were immersed for a further 18 hours in the 40-fold diluted PAP reagent, followed by further washing. Histochemical staining for peroxidase was carried out as before.

After histochemical staining, the sections were washed with water and post-fixed in osmium tetroxide solution (Palade, 1952) for 1 hour, before, washing again with water. They were dehydrated in graded alcohols and embedded in TAAB resin. Ultrathin sections (0.05 µm) were cut, mounted on uncoated 200 mesh copper grids and viewed without further heavy metal staining under the electron microscope.

To determine whether the immunocytochemical staining procedure was specific for T-H glycoprotein, control tests were carried out on the cryostat sections. Thus, in each of the four procedures mentioned above, the step involving application of anti T-H antibodies was either completely omitted or the latter antibodies replaced by normal sheep serum (diluted 1:10).

B) Use of Polyethylene Glycol (PEG) as embedding medium (as suggested by Mazurkiewicz and Nakane, 1972)

For this method, small pieces of perfused fixed hamster kidneys were dehydrated in graded ethanol and then
placed in 100% PEG for three changes (40 minutes each) in a vacuum oven at 40°C. Slices were then embedded in fresh PEG and allowed to harden at 4°C.

5 μm thick sections were then cut on a Spencer microtome and floated on a 5% glycerol solution in water for 1 - 2 hours. After this, the sections were transferred to egg albumin-coated slides and dried on a warming tray at 40°C for 30 minutes. The sections were then stained for peroxidase by the procedure of Burn et al. 1974 (as described for paraffin sections, see page)

After histochemical staining, the sections were washed in PBSA and temporarily covered with a glass coverslip using 90% glycerol in PBSA, and, examined under a light microscope for the quality of staining. For electron microscopic examination, the coverslip was removed and the sections washed in PBSA. After post fixation with Palade’s osmium tetroxide for 30 - 40 minutes, the sections were dehydrated and while still wet with 100% ethanol, a BEEM capsule filled with TAAB resin was inverted over each section and the resin allowed to polymerise at 60°C for 24 hours.

The hardened blocks were removed from the slides by immersion in liquid nitrogen and the tissue blocks lifted off. The desired areas were then trimmed and sectioned for electron microscopy.

C) The use of ultrathin sections

1) Peroxidase-labelled antibody method
   (modification of Kawaoi and Nakane, 1970 method)
   For this technique, hamster kidney slices, fixed by
perfusion in paraformaldehyde-glutaraldehyde or PLP, were used. After dehydration, slices were embedded in TAAB resin or Epon 812 (Luft, 1961). Ultrathin sections were then cut and collected on uncoated 200 mesh nickel grids. In order to facilitate the diffusion of antisera into resin, the sections were etched for 20 minutes with 10% H₂O₂. As a control for the etching process, some grids were floated on drops of distilled water for a similar period. After etching, the grids were thoroughly washed with PBSA and then placed face down on drops of rabbit (anti-hamster T-H)IgG for 30 minutes to 2 hours at room temperature, washed in several changes of PBSA (10 minutes) and placed on drops of peroxidase-labelled (sheep anti-rabbit) IgG for up to two hours. After thorough washing with distilled water, the sections were stained for peroxidase activity by incubating them in varying concentrations of 3,3'-diaminobenzidine substrate for 3 - 5 minutes.

For this, the grids were immersed into a solution, containing 12 - 25 mg/100 ml of 3,3' dianinobenzidine in 0.05 M Tris HCl buffer, pH 7.6 and freshly prepared 0.0025 - 0.01% H₂O₂. The solution was continuously agitated with a magnetic stirrer at 100 rpm. After incubation, the developed grids were removed and thoroughly washed with distilled water. Before viewing, the grids were post-fixed with 2% aqueous solution of osmium tetroxide (in order to stabilize and increase the electron density of the peroxidase reaction) followed by washing in water. The sections were observed under the electron microscope with and without further heavy metal staining.
2) **PAP Method** (Petrali *et al.* 1974; Erlandsen *et al.* 1974; Parsons and Erlandsen, 1974).

As in the peroxidase-labelled method, ultrathin sections which were collected on the 200 mesh unsupported nickel grids, were first etched for 3 - 20 minutes with an aqueous solution of 10% $\text{H}_2\text{O}_2$. The sections were then washed thoroughly with distilled water and in order to minimise the non-specific adsorption of antisera, they were treated for ten minutes with 10% normal sheep serum in Tris-buffered saline, followed by blotting, but no washing. The sections were immunocytochemically stained for T-H glycoprotein, by floating the grids for 3 - 5 minutes at room temperature on drops of solutions (placed on the glass coverslips in moisturised petri dishes), in the following sequence:

1) Rabbit anti-hamster T-H IgG, diluted 1:10 in Tris-saline, followed by washing in Tris-saline containing 1% normal sheep serum and blotting

2) Sheep anti-rabbit IgG, diluted 1:10 in Tris-saline, followed by washing in Tris-saline containing 1% normal sheep serum and blotting

3) Soluble PAP complex, diluted 1:40 in Tris-saline containing 1% normal sheep serum, followed by washing in Tris-saline but no blotting.

Peroxidase activity was revealed and the sections processed for electron microscopy by the same procedure as described before for the peroxidase-labelled technique.

**N.B.** Techniques B and C were tried in order to eliminate the problem of penetration of antisera into the tissue, which was
occasionally encountered when cryostat sections were used, and also to shorten, the rather long time required to stain latter sections. For example, it required at least 24 hours for the peroxidase-labelled antibody to penetrate into the tissue sections with a thickness of 20 - 40 μm, reproducibly. However, the results were not very successful, because, after incubation with the substrate, enough reaction product was deposited in a non-specific manner on the surface of sections so as to obscure the specific reaction sites. Even in those areas which escaped the non-specific deposits, the specific reaction sites were almost negative, indicating that the T-H glycoprotein had perhaps been denatured by the polymerization of the embedding medium.

**Adrenalectomy on Adult Hamsters**

Total adrenalectomies were performed on 12 adult hamsters (2 male and 10 female) and sham operations on four females. Of the former, both males and three females died immediately after the operation, either, as a result of internal haemorrhage or because of shock. All four sham-operated females survived the operation.

The female animals were preferred for these pilot experiments, as it was noticed that adrenalectomized females can survive for a comparatively longer period than males (survival period for males was approximately 6 days and that for females, 8 days).

Adrenalectomies were performed through the bilateral approach while the animals were still under sodium pentabarbital anaesthesia. After displacing the muscles and
viscera to one side, the kidney capsule was slightly ruptured at the place where the adrenal gland is attached to the kidney. The adrenal gland was then gently pulled apart and a tight suture placed at the junction of the adrenal gland with the kidney. After this, the adrenal glands were then carefully removed, muscles and viscera placed in their original position and the skin stitched back with the aid of a silk suture. The adrenal glands were immediately immersion-fixed in formal calcium chloride for routine histology.

Care was taken to avoid any unnecessary stress to the operated animals and if there was any internal haemorrhage, then such animals were killed immediately, by giving an excessive dose of anaesthetic. As far as possible, care was also taken to remove the adrenal glands intact to ensure that there were no remains left still attached to the kidneys.

After the operation, the animals were kept in Jencon's metabowls until killed and were fed on the standard hamster pelleted food, whilst their drinking water was replaced with 0.9% normal saline. In order to ensure that the animals were taking enough saline, their access to it was provided both, through an ordinary flask attached to the metabowl and by way of the thin rubber tubing which was attached to a 5 ml syringe filled with saline.

The kidneys were removed from the operated animals at intervals ranging from 4 to 8 days. The animals were anaesthetised and the kidneys perfused via the abdominal aorta with either:

1) Formal calcium chloride
2) Half strength Karnovsky's glutaraldehyde.

In some cases, after washing the kidneys with 0.1M sodium cacodylate buffer (pH 7.4), one kidney was tied off and immersion fixed in formal calcium chloride for use in fluorescence microscopy. The second kidney on the other hand, was perfusion fixed in \( \frac{1}{2} \) strength Karnovsky's glutaraldehyde and processed for routine electron microscopy, in order to check for any gross pathological changes which might have occurred because of the effect of adrenalectomy.
RESULTS
The Localization of T-H Glycoprotein in the adult Hamster Kidney

1) **By Immunofluorescence Microscopy**

In the kidneys of all the adult animals studied, the immunofluorescent staining was clearly and invariably localized in the ALH and DCT (Figs. 8, 9), but with the notable exception of MD (Fig. 10). The glomerulus (Fig. 10), PCT (Fig. 9) thin limb of the loop of Henle (Fig. 11) and CD (Fig. 11) also did not fluoresce.

Several different fixatives and fixation techniques were used and the results in all cases were similar in exhibiting T-H positive fluorescent staining only in the cells of ALH and DCT. The best results were obtained, however, with the material fixed by perfusion with formal calcium chloride. Regardless of the fixative used, in both ALH and DCT, the luminal border of the cells was always heavily stained for T-H. The amount of staining within the cells concerned, however, varied considerably, depending upon the fixative used. The kidney sections fixed by perfusion in formal calcium chloride for example, showed an almost uniform distribution of T-H over the cells of the ALH and DCT, except their nuclei (Figs. 8, 9, 10).

Immersion fixation with acetone and 80% ethanol, while yielding intensely and evenly stained reactive tubules, was found to be a less valuable procedure, because, the quality of fixation of the kidneys was, in general, not so good. Frequently, the tubules collapsed, resulting in a closed lumen (Fig. 12, 13).

The results obtained after perfusion fixation with PLP were comparable to those obtained with the formal calcium
chloride fixed material. Once again, the luminal border of the ALH and DCT was seen to be heavily stained for T-H glycoprotein. In the interior of the cells, on the other hand, staining was somewhat selective. The basal half of the cells of ALH and DCT for example, fluoresced brilliantly (Fig. 14). On close examination, small streaks of fluorescent material could be seen arising from the basal side of the cell and passing towards the lumen, suggesting the presence of T-H glycoprotein in the invaginations of the basal plasma membrane and/or perhaps also in the basally placed mitochondria. (The presence of T-H glycoprotein in the former was subsequently confirmed by immunoelectron microscopy - see later).

Results obtained with the paraformaldehyde-glutaraldehyde fixed material were variable. While the luminal border of the ALH and DCT still stained prominently, the distribution of T-H within the cells was somewhat patchy and it varied from cell to cell (Fig. 15).

Under the immunofluorescence microscope, the MD portion of the DCT was readily identified as an unstained area adjacent to the glomerulus (Fig. 10). The identification of non-fluorescent MD was facilitated by the use of a phase contrast attachment, when its narrow cells were shown to form a characteristic cushion like arrangement. In certain instances, the observations made by phase contrast microscopy were confirmed by a re-examination of the immunofluorescence preparations, which were subsequently stained with haematoxylin and eosin.

It was not possible by immunofluorescence microscopy to locate the junctions of the DCT with CD. Thus it cannot be
said with any degree of certainty on the basis of the results obtained with immunofluorescence microscopy alone, how far along the DCT T-H glycoprotein extends.

The cells of the CD were always found to be non-fluorescent (Fig. 11) and, therefore, lacking in T-H glycoprotein. Similarly, cells of the PCT too, did not show fluorescent staining in any of the kidneys examined (Fig. 9). They were identified by virtue of their prominent brush borders.

Immunofluorescence staining for T-H glycoprotein was demonstrated to be specific because in control sections, when the first layer (anti T-H antibody) was omitted in the indirect sandwich method used, no staining was observed in any part of the nephron.

In order to exclude the possibility of either induced or intrinsic fluorescence, several other tests were carried out. The results obtained showed that when fixed or unfixed air dried cryostat sections were viewed without any antibody treatment, varying degrees of dull green fluorescence sometimes quite marked, were observed in the brush border and sometimes even in the interior of the PCT cells. Fluorescence was not observed in the DCT, ALH and other tubules. In untreated fixed paraffin embedded material too, a slight amount of fluorescence was observed in the brush border of the PCT cells.

2) By Immunoperoxidase Techniques at the Light Microscopical Level

The examination of formal calcium chloride or PLP fixed paraffin was embedded sections which were subsequently treated
with the HRPO-conjugate prepared according to the two step procedure of Avrameas and Ternynck, failed to demonstrate T-H glycoprotein in the kidney sections.

Antigen was, however, readily demonstrated in the paraffin wax embedded 5 μm thick sections, stained with the technique finally involving the PAP reagent. The results agreed with those obtained by immunofluorescence microscopy. Diffuse overall staining of the cells of both ALH and DCT was obtained with 1:40 dilution of the PAP reagent. In formal calcium chloride fixed sections, the reactive cells usually had a granular, dark brown appearance (Fig. 16). The nuclei of these cells were readily identified as unstained clear areas surrounded by dark, well stained cytoplasm. The outer-medullary zone of the kidney revealed large areas of ALH and these were specifically stained for T-H glycoprotein. Similarly, in the cortex, cells of both the ALH and DCT stained intensely and contrasted markedly with the adjacent unreactive cells of the glomerulus and PCT (Fig. 16).

The tubular localization of T-H glycoprotein, obtained with the treatment of PAP reagent on the PLP fixed material was similar to that obtained with formal calcium chloride fixed kidneys. The luminal border of ALH and DCT stained heavily (Fig. 17). Inside the cells, however, staining was in some measure selective. The basal part of the cells showed an intense brown reaction product in the form of streaks which were very similar to those observed by immunofluorescence microscopy, perhaps again indicating an association of T-H with the infoldings of the basal plasma membrane and/or also perhaps with the mitochondria.
One feature, relating to the problem of penetration of antibodies into thick cryostat sections and also to the nature of the fixative used, was revealed, by the light microscopic examination of 1 μm thick araldite embedded sections which had been fixed in paraformaldehyde-glutaraldehyde and histochemically stained by utilizing conjugated antibodies. As is clearly demonstrated in fig 18, an intense staining for T-H glycoprotein was observed on the luminal border of the tubules. The interior of these tubules, on the other hand, completely lacked such staining. This suggests that either because of the thickness of the cryostat sections used, the antibodies were unable to penetrate into the cells thus resulting in the absence of intracellular staining or because of the destructive effect of the fixative used (in this case paraformaldehyde-glutaraldehyde), the chemical nature of the antigen had been altered.

In sections of that part of the DCT, where MD cells were cut and clearly identified, they were again found to lack the antigen (Fig. 18). It must be emphasised, however, that not all portions of the DCT which are seen in contact with the glomerulus are MD. For example, there are in Fig. 18 two sections of stained DCT apparently in contact with the glomerulus. One of these is uniformly stained all round its circumference and does not contain MD cells, because, the characteristically taller and narrower cells and consequently with more closely packed nuclei are absent. The second tubule, on the other hand, clearly contains identifiable MD cells, which are devoid of the glycoprotein. This underlines the importance of examining every stained
tubule in close proximity to the glomerulus, before making any statement about the presence or absence of T-H glycoprotein in the MD.

Another interesting feature was noticed: the intensity and quantity of luminal T-H staining in that part of the DCT near the MD was considerably less than in other portions of the DCT. (Fig. 18).

3) By Immunelectron Microscopy

a) The application of immuno-histochemical techniques to ultrathin sections

The attempts to stain the antigen in the ultrathin sections after araldite embedding were unsuccessful. In spite of some obvious positive reaction, the presence of a non-specific background staining made the interpretation of results extremely difficult. This method was therefore, abandoned.

b) The application of immuno-histochemical techniques to cryostat sections

All the results by immuno-peroxidase electron microscopy showed that while T-H glycoprotein in the hamster kidney was invariably associated with the cells of ALH and DCT (Fig. 19), it was always absent in the cells of MD (Fig. 20), PCT (Fig. 19), thin limb of loop of Henle (Fig. 21), CD (Fig. 22) and likewise in the glomerulus (Fig. 20). What follows is a detailed description of the results which have just been referred to in brief.

An examination of sections from kidneys which had been fixed by perfusion with paraformaldehyde-glutaraldehyde and
treated by any one of the two most successful procedures used for the demonstration of T-H at the electron microscopical level gave results showing the presence of T-H glycoprotein on the luminal border of the cells of ALH and the DCT (Fig. 19). In sections, cut very close to the surface of 10-20 μm thick cryostat sections, however, the reaction product was also occasionally found in the basal plasma membrane and its infoldings. (Fig. 23, the experiments described later showed that this is indeed the case). This indicates that with this particular technique, the lack of reaction in the infoldings of the cells of ALH and DCT in deeper sections was probably due to the lack of penetration of antibodies. Similarly, the amount of precipitate deposited on the luminal surface of these cells also varied inversely with the thickness of the cryostat sections used.

T-H glycoprotein was found to extend along the DCT up to its junction with the CD. This junction can be seen in Fig. 24, by the change in ultrastructure of the cells: those of the DCT have their elongated mitochondria arranged roughly perpendicular to the base of the cell. On the other hand, cells of the CD may be seen to be extremely vacuolated and to have their small mitochondria randomly distributed.

The most consistent and informative results were obtained with the material which had been fixed by perfusion with the PLP fixative. Again, T-H glycoprotein was found to be exclusively associated with the cells of ALH and DCT and other cell types were negative. Within the cells concerned, the greatest amount of reaction product was seen on the luminal border and regardless of the thickness of cryostat sections used, also in association with the basal plasma
membrane (including its infoldings) and the lateral membranes - the total plasma membrane system in fact (Figs. 25, 26). Referring back to the immunofluorescence and other light microscopical findings (see page 71), it is now clear that the reaction product seen in the basal part of the cells, is actually associated with the basal plasma membrane and its infolding and not with the mitochondria. Occasionally, it appeared that Golgi bodies (Fig. 27), and cisternae of the endoplasmic reticulum (Figs. 25, 27), also contained the glycoprotein.

The absence of T-H in the cells of MD (as observed by immunofluorescence and light microscopical immuno-histochemistry) was confirmed by the immuno-electron microscopical techniques. Figs. 20 & 28 clearly show the cells of the MD with their closely packed nuclei and it can be seen that there is no reaction either on the luminal border of these cells or in their intracellular membranes. On the other hand, the plasma membrane system of the cells of DCT opposite to the MD are positive for the T-H specific reaction.

A diagramatic representation of the presence of this glycoprotein in a single nephron of the hamster kidney is shown in Fig. 29.

In control sections, no reaction was seen in any tubule or glomerulus.
The Localization of T-H Glycoprotein in the Human Kidney

1) By Immunofluorescence Microscopy

The distribution of T-H glycoprotein in surgically removed normal pieces of adult human kidney was precisely similar to that found in the adult hamster. In addition, when casts were present, they also reacted strongly for T-H glycoprotein.

Distinct specific fluorescent staining was confined to the medullary as well as cortical ALH (Figs 30, 31) in addition to DCT in the cortex (Fig. 32). Once again, cells of the MD were found to be negative (Fig. 33). As in the hamster kidney, glomeruli, PCT, thin limbs of loop of Henle, and CD were also negative. As described before, anti-hamster T-H antibodies when applied to the human kidney sections, gave comparable results to those of anti-human T-H antibodies, confirming that these two antibodies cross-react with each other.

Both, the cell interior as well as the luminal border of the cells of ALH and DCT gave brilliant but diffuse fluorescence. The nuclei of these cells, however, were always unstained.

To exclude the possibility of non-specific binding of antibodies, control tests were carried out by omitting the first layer (anti-T-H antibody) from the indirect procedure used for localization. No fluorescence was observed in these tests, suggesting that the results described above were specific.

2) By Immunoelectron Microscopy

As in the hamster, once again, T-H in the human kidney
was clearly localized in the ALH (Fig. 34) and DCT (Fig. 35) but with the notable exception of the MD (Figs. 36 A & B). Cells of the PCT (Fig. 34), thin limb of loop of Henle, CD (Fig. 36A) and likewise those of the glomerulus lacked the immuno-peroxidase reaction.

In the cells of ALH and DCT the reaction was confined only to the membrane system. Thus, the luminal border of the cells of these tubules together with the lateral and basal plasma membrane (including the infoldings of the latter) stained intensely for T-H glycoprotein (Figs. 34, 35). The immunocytochemical reaction was not detected in mitochondria and nuclei. No evidence for the presence of T-H was observed in the membranes of the cells of MD.

The reaction for T-H was found to be specific. No staining was observed in the control tests done by omitting T-H antibodies from the procedure used to localize this glycoprotein.
The Ontogenic Development of T-H Glycoprotein in the Hamster Kidney

Metanephric kidneys can be clearly seen and extracted in hamster foetuses of 10 days gestation and beyond. Light microscopy of renal medullary areas in the 11 day foetal kidneys showed a poorly organized, loose mesenchymal stroma with very few recognizable tubules except for the CD which were quite abundant in the deeper medullary zone. On the 12th day of gestation, a few glomeruli were also observed. The distal tubules which later becomes differentiated into ALH and DCT were abundant and easily recognizable within the mesenchymal tissue as rays extending from the medulla. By day 14, PCT were also readily definable.

1) By Immunofluorescence Microscopy

T-H glycoprotein was not demonstrated in the foetal hamster kidneys sacrificed on the 10th and 11th day of gestation, but positive staining was first observed on the 12th day. Kidneys from foetuses studied on this day showed some patchy but specific apple green fluorescent staining within the medullary distal tubules (Fig. 37, By distal tubule is meant here that part of the foetal nephron which ultimately differentiates into ALH & DCT). No staining was observed in the presumed cortical area. On day 14, distal tubular cells in the medulla showed definite fluorescence for T-H glycoprotein in all the foetal specimens studied (Fig. 38). Traces of positive staining were also observed in the cortico-medullary region (Fig. 39). On day 15, the number of positively stained medullary tubules was considerably
more than on day 14. Thus, rays of stained tubules could easily be seen extending from deep in the medulla toward the cortical region (Fig. 40).

In all the foetal kidneys studied, regardless of the fixative used, the luminal border of distal tubules fluoresced more brilliantly than the cell interior—nuclei being always negative. In fact, in the distal tubular cells of 12 and 14 days foetal kidneys fluorescence was observed only on the luminal borders (Fig. 37, 38). After birth, the intensity, number and extent of the stained tubules increased progressively. On the 16th day (the first day post-partum), staining was observed both within the cortex and medulla (Fig. 41). Medullary tubules, however, fluoresced more brilliantly than those in the cortex.

By the 3rd day after birth, it was possible to identify ALH as separate entities, and these were also positive (Fig. 42). On this day, as in the adult, MD were identified and found to be negative (Fig. 43), although, adjacent DCT cells contained T-H glycoprotein. By the 23rd day after birth, the intensity of fluorescence staining, approximated to that seen in adult kidneys (Fig. 44). In foetal as well as neonatal kidneys, all tubules other than ALH and DCT, were negative.

Immunofluorescence staining for T-H glycoprotein in the foetal and neonatal hamster kidneys was specific. Control sections did not stain.

2) **By Immunoelectron Microscopy**

The localization of T-H glycoprotein in the foetal and neonatal hamster kidney by immunoelectron microscopy was
similar to that obtained by immunofluorescence microscopy.

No staining was observed in kidneys of foetal hamsters sacrificed on the 10th and 11th day of gestation. Positive staining for T-H was first noticed in the kidneys of 12 day foetuses. The reaction on this day was confined, only to the undifferentiated distal tubules present in the medullary region (Fig. 45). Regardless of the fixative used, the reaction within distal tubules on this day was restricted to the luminal border. Cytoplasmic organelles, lateral and basal plasma membranes (including its invaginations) were negative.

The intensity of T-H positive staining was slightly more in the distal tubules of 13 days foetal kidney (Fig.46). Proximal tubules identified by their prominent brush border were found to be negative. Similarly, cells of the CD and mesenchymal tissue also did not show the reaction (Fig.46). In the PLP fixed material, a slight amount of scattered HRPO-staining was also occasionally observed in the basal invaginations of the plasma membrane and possibly the endoplasmic reticulum (Fig. 47). It was not until the 15th day of gestation that stained distal tubules were also observed in the outer cortex.

The immunohistochemical reaction became more prominent in the cortical region during the first few days after birth. On the 4th day post partum, a strong but specific reaction was present in the DCT and in ALH. In the paraformaldehyde-glutaraldehyde fixed neonatal kidneys, a HRPO-positive reaction in the form of a black precipitate was observed on the luminal border as well as in some of the invaginations of the basal plasma membrane. In the PLP fixed
neonatal kidneys, on the other hand, the reaction product was found to be associated with the luminal border, lateral membranes, invaginations of the basal plasma membrane and perhaps to the endoplasmic reticulum of the cells of DCT and ALH (Fig. 48).
The Localization of T-H Glycoprotein in the Adrenalectomized Hamster Kidney

As described earlier, the urinary T-H glycoprotein is, in the hamster and human, associated with the plasma membranes of the cells of ALH and DCT, with the notable exception of the MD.

It thus appears that T-H glycoprotein is confined only to that part of the nephron responsible for the process of urine dilution (see discussion). As this function is at least in part regulated by adrenal cortical hormones, the effect of adrenalectomy on the distribution of T-H has therefore, been studied in pilot experiments done on hamsters.

When the immunofluorescence technique, used to localize T-H glycoprotein staining in the normal hamster kidney was applied to the kidneys of adrenalectomized animals, a varying degree of reduction in the amount of T-H was observed, initially from the DCT and later from the ALH. These changes were frequently accompanied by the appearance of brilliantly fluorescing casts.

In the first hamster, which was killed 4 days after adrenalectomy, the effect could already be seen. For example, cells of some of the DCT's clearly showed a marked reduction in the amount of T-H glycoprotein-specific fluorescence (Fig. 49). In some of the ALH and DCT's, casts were observed which were strongly T-H positive, the cells themselves were lacking T-H glycoprotein staining (Fig. 50). Such an effect was not, however, observed in all
the ALH cells examined. Most of the ALH tubules (especially in the inner medulla), for example, still fluoresced brilliantly and were thus, in no way different from those of normal control animals. This indicates that the effect of adrenalectomy in this animal was restricted only to certain regions. In those tubules which showed no reduction of T-H, all the cells (except their nuclei) fluoresced brightly.

The cells of the affected ALH and DCT tubules, on the other hand, showed varying degrees of reduced fluorescent staining. Under high power, such a disappearance of specific staining in the affected tubules was often seen in the basal portion of the cells (Figs. 49, 50). The MD cells were always negative as were glomeruli, PCT and CD cells.

Similar results were obtained from the hamsters sacrificed on the 6th, 7th and 8th day after adrenalectomy. The effect of adrenalectomy in relation to the loss of T-H specific staining was, however, considerably greater at the 6th, 7th and 8th day post-adrenalectomy. The cells of most of DCT in the 6 days adrenalectomized animal, for example, completely lacked fluorescent staining (Fig. 51). It made no difference where these tubules were located in the cortex. The presence of brilliantly fluorescing casts in these tubules suggests that the glycoprotein has been gradually peeled off from the surface of ALH and DCT cells and begun to accumulate in the tubular lumen, instead of being flushed out in the urine. A slight loss of staining was also observed in the cells of the ALH of the outer medulla (Fig. 52).

The loss of T-H was even more apparent in the 8 days adrenalectomized hamster. Fluorescent staining for T-H
glycoprotein disappeared almost completely from within the cells of ALH and DCT (Figs. 53, 54). Furthermore, almost all the tubules were found to be blocked with fluorescent casts.

The results from sham-operated control animals were comparable with unoperated normals. No casts were observed and the cells of both ALH and DCT fluoresced brilliantly (Fig. 55).

N.B. At the time when these experiments involving hamster adrenalectomy were carried out, the advantages of PLP as a fixative for use in the demonstration of glycoprotein by immuno-enzyme techniques had not been appreciated. The localization of T-H glycoprotein in the adrenalectomized hamster was, therefore, observed only by fluorescence microscopy. However, investigations on the effect of adrenalectomy on T-H glycoprotein has been carried out at the electron microscopical level in rats, and these will be described later (see addendum).
The Light and Electron Microscopical structure of the Adrenalectomized Hamster Kidney

An examination of haematoxylin and eosin stained preparations of kidneys for light microscopy of adrenalectomized animals showed (except for the presence of casts in the DCT and ALH), and apparently normal histology.

The ultrastructure of various portions of the nephron and CD from the kidney of a hamster killed 8 days after adrenalectomy were also studied. It was compared with that of the tubules from the normal hamster kidney, in order to see if there had been any pathological changes, which would not have been revealed by light microscopy.

There were no significant alterations observed in the ultrastructure of any important cell organelles (e.g. mitochondria, Golgi body, lysosome etc.), which would suggest serious damage to the tubular system. The overall fixation of the cells was, however, not as good as in controls; possibly because the casts present in the tubular lumina made it difficult for the perfusion fluids to penetrate deep into the cells.

Proximal convoluted tubule

The lumen of the PCT was wide open with a well developed brush border (Fig. 56). The basal plasma membrane exhibited numerous infoldings, between which elongated mitochondria were present. The size and shape of the mitochondria and the arrangement of their cristae compared favourably with those of the normal kidney (Fig. 57). Cells of the PCT contained abundant cytoplasm along with the centrally placed, large and spherical nuclei. Well developed Golgi bodies were
present in supranuclear as well as in lateral positions. A few lysosomes and microbodies (peroxisomes) were also observed.

**Thick ascending limb of loop of Henle**

As in the normal controls, the cells of the ALH in the adrenalectomized kidney, were found to be of irregular shapes and sizes. The cells of the cortical part of the thick limb for example, were much thinner in comparison with those in the medulla. Both in the cortex as well as in the medulla, mitochondria were regularly arranged within the interdigitations of the basal plasma membrane (Fig. 58).

The luminal cell membrane with its few short microvilli was found to be in no way different from that of the normal kidney (Fig. 59). The flattened nucleus occupied almost the entire height of the epithelial cell. The Golgi apparatus with its paranuclear position as well as other cell organelles (RER, lysosomes, etc.) appeared normal.

**Distal convoluted tubule**

Once again, as in the normal controls (Fig. 61), the cells of the DCT of the adrenalectomized hamster kidney were seen to be regularly interdigitated with deep, parallel and vertical infoldings of the basal plasma membrane, amongst which were packed long and slender mitochondria. Some of these infoldings were, however, found to be extremely dilated (Fig. 61) and thus, formed large empty looking spaces in the basal part of the cell - the spaces, presumably fluid filled in life, could be a consequence of physiological changes resulting from adrenalectomy. Apart from this, no other
changes in the ultrastructure of the cells of DCT were noticed.

**Collecting duct**

The cells of the CD of the adrenalectomised hamster kidney (Fig. 62) resembled more or less to the similar cells of the normal hamster kidneys (Fig. 63). The cells lining these excretory ducts were usually low cuboidal with regular cell borders. Small and short interdigitations of the basal plasma membrane and distribution of the few scattered organelles, was also in no way different from that seen in the normal kidneys.
Light and Electron Microscopical Observations on the Macula Densa of the Distal Convoluted Tubule of the Hamster Kidney

The chances of observing a clearly transverse cut of a DCT at the level of MD in ultrathin sections were very small. Therefore, a large number of sections had to be scanned even to see a significant number of MD, since they constituted such a very small proportion of the total tubular epithelium.

Fig. 64 is a photomicrograph of a 1.0 μm araldite section, showing a MD and associated DCT cells and Fig. 65 is low power electron micrograph of a comparable region. In the latter, an MD is shown to the right and it is separated from the rather flattened cells of the extraglomerular mesangium by a basal lamina, which appears to be continuous.

Certain characteristic features of the MD and its cells become immediately apparent from an examination of the above figures.

a) The MD formed a distinct cushion
b) Its nuclei and cells were more tightly packed than in the remaining part of the DCT.
c) The cells were distinctly columnar in shape
d) The mitochondria had predominantly circular or elliptical profiles and were quite numerous in the apical cytoplasm, whereas in the cells of DCT, they were usually elongated and occurred in parallel arrays, primarily in the basal part of the cytoplasm.
e) There were cells marked by arrows (Fig. 65), occupying a short transition zone on either side of the MD in which the arrangement and
form of the mitochondria was intermediate between the typical MD cells and the cells of the DCT. The cytoplasm of these transition zone cells was marginally less dense.

f) 'Light' and 'Dark' cells described by some workers in other species were not observed in the hamster kidney MD.

In some of the examples studied there were regions where the epithelium appeared to be more than one cell layer in thickness (Fig. 66). An examination of a much larger number of stained 1.0 μm araldite sections and 5.0 μm paraffin sections, however, suggested that when the plane of the section was precisely transverse, the epithelium of the MD was only one cell layer in depth (Fig. 64).

Further features distinguishing MD cells from neighbouring DCT cells became immediately evident in electron micrographs at higher resolutions and these are described in what follows.

The cell surface and subadjacent region

The luminal plasma membrane was associated with a mucoprotein coat thus forming a fuzzy coating, the glycocalyx. This mucoprotein coating in common with that of all DCT cells stained blue with alcian blue after the periodic acid Schiff/alcian blue technique. This was in contrast to the glycocalyx of the PCT cells which stained magenta. There was, however, one very important difference and this was, as described before, the total absence of T-H glycoprotein in the MD of the hamster kidney, in comparison with that which
occurred consistently elsewhere in the DCT and in the ALH.

Two types of projections could be seen arising from
the luminal plasma membrane of MD cells:

a) regular, long, finger-like microvilli which
were sometimes branched (Fig. 67), and
b) short, blunt and rather irregular tubulo-
vesicular folds (Fig. 68).

Parallel bundles of microfilaments were present only
in the first type, while the second type contained varying
degrees of small and large vesicles. Vesicles containing
folds were rarely seen in the DCT and ALH. Also, the
average number of microvilli and tubulo-vesicular folds
per cell in the MD was more than in the ALH and DCT. Singly
distributed and scattered cilia were also seen occasionally
in the MD (Fig. 67).

In almost all the cells of the MD, the subsurface
cytoplasm was filled with numerous small membrane-bounded
vesicles of circular profile (Fig. 69). Such vesicles were
also seen in the FCT, DCT and ALH, but differed in being much
less frequent and generally more scattered. The vesicles
present in the surface folds and the subsurface cytoplasm,
both appeared to be derived from the invaginations of the
plasma membrane (Fig. 69).

The lateral and basal plasma membrane

The plasma membranes of adjacent cells interdigitated
to varying degrees (Fig. 70) and in this respect
were not significantly different from those of ALH and
the rest of DCT. At the luminal margins, adjacent
membranes formed **zonulae occludentes** or 'tight junctions' (Fig. 69) which extended basally for a short distance. These junctions were found to be in no way different from those seen in other parts of the nephron. The configuration of the basal plasma membrane, which rests on a basal lamina, was however markedly different from other regions of the nephron. There were numerous and complex infoldings of the basal plasma membrane but they were unlike those of the DCT (Figs. 68, 70) in two important respects:

a) They were restricted to the basal part of the cell, and

b) lacked any distinct orientation.

Occasionally, cell processes with numerous small vesicles were observed adjacent to the cell base (Fig. 68). These processes were completely enclosed by a plasma membrane. At certain places, small vesicles were also been in contact with the basal plasma membrane (Figs. 68, 71).

Contrary to some reports on other species, the basal lamina in the MD of the hamster appeared to be continuous throughout (Fig. 71).

**Ribosomes and endoplasmic reticulum**

Rosettes of ribosomes (Polyribosomes), so characteristic of the DCT and the ALH, were relatively less in the MD cells. Whenever seen, they were randomly distributed throughout the cytoplasm (Fig. 71). Similar observations were made about the rough endoplasmic reticulum which was not found to be restricted to any particular region of the cell. It comprised apparently unconnected stretches of variable shapes and lengths and its cisternae were quite commonly dilated (Figs. 70, 71). In contrast to the PCT cells (Fig. 57), an extensive cisternal
system and large aggregations of smooth endoplasmic reticulum were never observed. Single membrane-bounded vacuoles of variable sizes and devoid of ribosomes were scattered throughout the cytoplasm (Fig. 72). These were unlike the elements of both the smooth endoplasmic reticulum of the PCT and of the subsurface vacuoles.

Golgi complex

Light microscopy was used specifically to study the position of the Golgi body in a relatively large sample of MD than is practicable with the electron microscope.

The results, using metallic impregnation technique were disappointing. The osmium tetroxide method failed to demonstrate the organelle at all. Aoyama’s technique on the other hand, gave excellent results in the cells of the glomeruli and all tubules except the DCT, ALH and MD. Here although doubtless, the Golgi area was impregnated, there was so much general precipitation in the cytoplasm that it was not possible to differentiate it from the strongly argentophil background. It was noted, however, that the Golgi bodies of the cells of the PCT and CD generally formed a perinuclear ring at approximately the level of the equator of the nucleus; they were only very occasionally seen in an unequivocally infranuclear position, in the cells of PCT.

Electron microscopy, however, supported the view that as in the PCT, the Golgi complex in the DCT and the ALH comprised separate groups of lamellae, vacuoles and vesicles surrounding the nucleus and these were normally to be found in the upper half of the cell. This position is shown in Fig. 73, in a cell of the ALH. Some of the MD cells also showed a comparable location
of the Golgi elements (Fig. 74). In this instance, two profiles of a ring-like configuration of Golgi elements are shown one on each side of the nucleus. However, this is by no means always the case in the MD. In Fig. 67, for example, only one of Golgi complex is seen to the top left hand side of the nucleus - there being no matching component on the opposite side. In MD cells there were other configurations which were not observed either in the cells of ALH or DCT proper. One example is shown in Fig. 68, where profiles of two Golgi elements are shown in the same cell; one clearly infranuclear in position and the other lateral to the nucleus, somewhat above the equator. Second and third examples are illustrated in Fig. 75, where the cell to the left shows one Golgi profile in an infranuclear position and the cell to the right shows three discrete Golgi complexes, all in infranuclear positions.

Mitochondria

The intracellular distribution of mitochondria constituted a very significant morphological difference between the MD cells and those of other nephric tubules. In contrast to the arrangement of mitochondria in the DCT, where large and elongated mitochondria occupied positions in between perpendicular invagination of the basal plasma membrane (thus, dividing the basal half of the cell into several compartments, Fig. 60); in MD cells, mitochondria were not enclosed within the folds of the basal plasma membrane. They were much smaller in size, usually had circular or oval profiles and were evenly distributed throughout the cell (Figs. 68, 72, 74, 76).

The cristae mitochondriales were lamellate in form
This was in contrast to the PCT cells where circular profiles were not uncommon (Fig. 57) and ALH and DCT where anastomoses of cristae as well as circular profiles were quite frequent (Fig. 60).

**Lysosomes, peroxisomes and other intracellular granules**

Histochemical techniques both at the light and electron microscopic levels showed the presence of acid phosphatase containing bodies or lysosomes in the CD (Fig. 77), PCT (Fig. 78) ALH, DCT (Fig. 79) and cells of the MD (Fig. 80). Only in PCT and CD cells, however, could it be said that these organelles were a constant and frequent feature. In MD cells, on the other hand, they were sporadic and of variable sizes, but even so probably marginally less frequent than in the ALH and DCT.

Peroxisomes (microbodies) as demonstrated cytochemically for the catalase which they contain, although, abundant in PCT cells, appear to be totally absent elsewhere in the tubular system including PVID cells of the hamster kidney (Fig. 81).

Finally, a class of granular bodies sparse in their distribution was observed in the cells of MD. They were smaller than the lysosomes mentioned above and resembled the so called electron-dense homogeneous cytosomes of Tisher et al. (1968) and non-specific granules (stage I and II) of Biava (1967). In the hamster, these granules possessed a dense and compact matrix which was separated by a less opaque area from the single enveloping membrane (Fig. 70). It can be confidently asserted on the basis of morphological and cytochemical grounds that they were not peroxisomes, but
whether or not they contained acid phosphatase and were thus lysosomal in nature has proved difficult to determine with any degree of certainly. They tended to occur in the proximity of the basal plasma membrane, but not exclusively so.

**Nucleus**

Nuclei in the MD cells, as stated previously, were closely packed together and were generally centrally placed. Most of the nuclei were characterised by the presence of rather large masses of heterochromatin in a peripheral position adjacent to the nuclear envelope (Fig. 65). Similar nuclei also occurred, but more sporadically in the rest of the DCT and in other parts of the tubular system as well. This feature was sufficiently consistent to form an additional diagnostic characteristic of the MD at the light microscopical (Fig. 64) as well as at the electron microscopical level (Fig. 65).

**Multivesicular bodies**

Multivesicular bodies, varying greatly in size and number were observed in the ALH, the DCT and the cells of MD. The majority of them were composed of light background matrix within which small vesicles were present (Figs. 69, 75, 76). Some of them, however, possessed a more electron dense background matrix.

**Cytoplasmic ground substance and microtubules**

In the cells of the MD, the cytoplasmic ground substance was composed of a thin flocculent material containing the organelles described. The density of this substance was almost equally uniform in all the cells, so that it was
found difficult to differentiate between the so called 'light' and 'dark' cells.

A small number of microtubules were also observed. In some instances they were situated in the perinuclear cytoplasm (Fig. 71) in small groups, otherwise, they were randomly distributed. In ALH cells and the rest of DCT, only occasional isolated examples were noted.

'Dark' and 'light' cells

'Dark' and 'light' cells usually found in the DCT and the CD, were not observed in the MD of the species studied. The distribution of various organelles as described above was almost uniform in all the cells of the MD. However, as mentioned before, on either side of the MD proper, possibly lay a short transitional zone, the cells of which possessed slightly less dense cytoplasm. The number of cells in such transitional zones was usually one on either side (Figs. 65, 66). The arrangement and form of mitochondria was intermediate between typical MD cells and the cells of DCT and ALH, and were not enclosed within invaginations of the basal plasmalemma. The Golgi apparatus was always supra-nuclear in position.

Extraglomerular mesangium

The cells of the extraglomerular mesangium (sometimes called Polkissen, Lacis cells or polar cushion) formed a closely packed cushion of cells underneath the MD between the walls of the afferent and efferent arterioles (Fig. 65).
An Evaluation of the Fixatives and Fixation Techniques in Relation to the Immuno-Cytochemical localization of T-H Glycoprotein

1) The Comparison of Fixatives and Fixation Techniques

Although, earlier workers have frequently used unfixed cryostat sections in immunofluorescence microscopy, fixed tissue was preferred in the present investigations. It was found that the use of unfixed material greatly increased the difficulty of histological interpretation. Such tissue was also frequently found to possess intrinsic fluorescence.

The best results were obtained with the material fixed with formal calcium chloride or PLP reagent.

A) Immersion fixation

In general, the quality of preservation of the tissue by immersion was poor. As observed both by light and electron microscopy, only tubules located immediately underneath the renal capsule showed patent tubular lumina. The cells of even these superficially located tubules were found to be poorly fixed, especially with dilute (weak?) fixatives like PLP.

However, when paraformaldehyde-glutaraldehyde or karnovsky's fixatives were applied to the exposed kidney surface in vivo, the fixation of PCT cells located within a 1-2 mm thick superficial zone was found to be satisfactory. The brush border of these cells was regularly arranged and most of the mitochondria being perpendicular to the basement plasma membrane. Lumina of the ALH and DCT tubules on the other hand, were usually collapsed to a varying extent. Similarly, the extracellular spaces, both,
between the lateral cell membranes and the tubules themselves were quite frequently increased. At the same time, it was demonstrated by electron microscopy that the mitochondria in ALH and DCT have lost their regular arrangement and became more randomly distributed. The preservation of other organelles and the cytoplasm was also poor and this made it extremely difficult to identify various portions of the nephron. Greatly enlarged and deformed ('exploded') cells with badly damaged organelles were a common feature.

B) Vascular perfusion

The overall preservation of kidneys fixed by vascular perfusion was superior to that by immersion. The success of perfusion fixation, however, depended on various factors. The complete elimination of blood from the vascular system by rinsing the kidneys through with a buffer solution, was found to be an important factor in facilitating a good perfusion with fixative. In this context, the use of heparin in the rinsing fluid was found to be very useful. Various concentrations of heparin were tried and the best results were obtained when 500 units of heparin were added to each 100 ml of the rinsing fluid.

Also, the addition of fast green to the fixative as a built-in-marker, proved to be very helpful in judging the effectiveness of the perfusion. Thus, well perfused kidneys had macroscopically a uniform light green colour. Poorly perfused kidneys, on the other hand, showed a spotty surface in the form of red and green patches.
Light and electron microscopical examination of soft and poorly perfused kidneys usually revealed grossly distorted tissue morphology. Such kidneys were usually discarded. However, when it was not practically possible to use another animal, only hard and green portions of such poorly fixed kidneys were used for further analysis.

In order to see the effect of the temperature of the perfusion fluid upon the preservation of the tissue, a series of initial experiments were done in which kidneys were perfused with fixatives either chilled (4°C), heated (37°C) or at room temperature (20-23°C). No obvious light or electron microscopic differences were noticed between the material fixed at 4°C, or at room temperature. Although, the rate of diffusion of fixative improved significantly at 37°C, the material fixed at this temperature was found to be unsuitable for the present studies because of the possible risk of extraction of T-H glycoprotein.

The best results were obtained when perfusion of the fixative was done retrograde through the dorsal aorta (perfusion method 1) although, administration of the fixative via the heart (perfusion method II) also gave satisfactory results.

Kidneys fixed by vascular perfusion showed much better preservation than those fixed by immersion. Also, the rate and depth of penetration of the fixative were greater by this method and the time needed for adequate fixation was also reduced. For example, in order to get a satisfactory fixation by immersion alone, the tissue had
to be left in the fixative for at least 12-18 hours, while 5-10 min. fixation by perfusion followed by further fixation by immersion for 2 hours gave much better results. The increased rate of penetration coupled with better preservation by perfusion is probably related to the fact that the fixative is able to reach the cells both through the vascular supply and also through the tubular lumina (via glomerular filtration system?)

One further advantage of vascular perfusion was noticed. The tissue fixed with this technique was comparatively much harder and this helped in further handling of the tissue with minimum damage.

The best fixation by perfusion was obtained when the perfusion pressure was increased to 140 mm mercury. It was noticed that a perfusion pressure similar to that of normal blood of the animals, usually resulted in an incomplete fixation of kidneys, especially the medulla.

2) The Effect of Different Fixatives on Tissue Preservation and its Relation to the Immunofluorescent Staining for T-H Glycoprotein

A) Unfixed cryostat sections

Because of the extremely poorly preserved morphology which made the identification and histological interpretation of different portions of nephrons very difficult, unfixed cryostat sections were found to be unsuitable for the localization of T-H glycoprotein by fluorescence microscopy.

Also, when anti-T-H glycoprotein antibodies were used
at a dilution of 1:10 (as used with the fixed material), the results were difficult to interpret because of a heavy diffuse background staining over the whole section. Furthermore, even the intensity of specific fluorescence in the stained tubules was variable, with some tubular cells showing a strong reaction while others scarcely stained at all. This lack of clear demarcation was not apparent in the fixed paraffin sections.

B) **Fixed paraffin sections**

A far clearer picture overall was obtained with fixed tissue. With all the fixatives employed, the cellular morphology was better preserved than in the unfixed cryostat sections. T-H positive tubular cells were easily and precisely identified. The best histological details were, however, obtained in kidneys fixed either in paraformaldehyde-glutaraldehyde, although, PLP and formal calcium chloride material also gave satisfactory results.

The fluorescent staining in PLP and formal calcium chloride fixed material was intense and provided a sharp contrast with the surrounding negative cells. Both, the cell interior and the luminal border of ALH and DCT (except MD) fluoresced brilliantly when these two fixatives were used. (Figs. 8 to 11, 14 and 30 to 33). In paraformaldehyde-glutaraldehyde fixed material on the other hand, while the luminal border fluoresced, almost negligible staining was obtained inside the cells (Fig.15).
3) The Effect of Fixation on the Localization of T-H Glycoprotein in Immunelectron Microscopical Preparations

A) Aldehyde fixation

The ultrastructural details required for the immunocytochemical work were well preserved when paraformaldehyde or paraformaldehyde-glutaraldehyde was employed for tissue fixation by perfusion. Satisfactory preservation was obtained with 2% and 4% paraformaldehyde when perfused kidney slices were left for further fixation by immersion of up to 18 hours. Fixation for shorter periods were found to be inadequate and usually led to swelling. Similarly, in the kidneys fixed for 4-8 hours with 1% paraformaldehyde, tissue swelling was a common occurrence. Most of the cytoplasm was extracted, thus leaving large intracellular spaces. Immersion fixation of similar periods after perfusion with 2% paraformaldehyde plus 0.5% glutaraldehyde provided good preservation of the tissue.

Although, the general preservation of the tissue was reasonably good with all the fixatives mentioned, the immunological reaction for T-H was not very favourable. When 40 um thick cryostat sections obtained from the material fixed as above were treated with the antibodies, and processed for electron microscopy, the electron dense precipitate of oxidised 3-3' dianinobenzidine was localized mainly on the luminal border of the cells of ALH and DCT while the interior of these cells was generally negative (Figs. 18 to 22). Occasionally, however, other parts
of the membrane system (lateral cell membranes and infoldings of the basal plasma membrane) showed weak and scattered staining (Fig. 23). It should be pointed out that the positive reaction just referred to was only observed close to the cut surface of the thick cryostat section. Similar cells present in the deeper portions of these sections were either totally negative or unevenly stained. The problem of penetration was less common when the thickness of the cryostat sections was reduced from 40 μm to 10 μm. The number of tubules (ALH and DCT) with positive cells was much higher at this thickness.

Fixation of tissue in 1 and 2% paraformaldehyde yielded the optimum intensity of the reaction product; higher concentrations (4% paraformaldehyde or 2% paraformaldehyde plus 1% glutaraldehyde greatly reduced the intensity of the reaction.

B) Periodate-lysine-paraformaldehyde (PLP) fixation

Preservation of the ultrastructure was satisfactory when this fixative was employed. The composition of PLP which resulted in the best preservation of ultrastructure was 0.01M sodium phosphate buffer, pH 7.3. At this concentration membranes, ribosomes, mitochondria and cytoplasm were all well preserved. The quality of preservation with this fixative compared favourably with that obtained with ½ strength Karnovsky's glutaraldehyde and was much better than that obtained with either 4% paraformaldehyde alone or 2% paraformaldehyde and 1%
glutaraldehyde. However, when the concentration of the
paraformaldehyde in the PLP fixative was reduced to 1%,
the overall preservation of tissue was not as good.
Although, membranes at this concentration were still well
preserved, there was some extraction of cytoplasm and
other organelles (Fig. 28).

The localization of T-H glycoprotein in the hamster
and human kidney was excellent when this fixative was used.
The reaction product was clearly localized on the luminal
border as well as in the lateral membranes and invaginations
of the basement membrane of the cells of ALH and
DCT (Figs. 25, 26, 34, 35).
DISCUSSION
The homogeneity of T-H glycoprotein

Since the main objective of this thesis was to localize T-H glycoprotein at cellular levels in hamster, rat and human kidneys, the first question which automatically comes to mind is: How can one be sure that the substance demonstrated by the techniques used is indeed a specific glycoprotein?

T-H glycoprotein which is quantitatively an important constituent of normal urine, can be readily prepared by repeated salt precipitations, thus yielding a material of relatively constant physical properties and chemical composition (Fletcher et al. 1970c). The glycoprotein prepared from hamster urine by salt precipitation on the basis of its insolubility in 1.0M NaCl was shown by earlier workers (Dunstan et al. 1974) to be of homogeneous nature. In the present studies, antigenically pure urinary glycoprotein (T-H glycoprotein) was prepared by successive salt precipitations - that of hamster with 1.0M NaCl and that of man with 0.58M NaCl solutions. The glycoprotein preparations obtained as above were considered to be as free as possible from other contaminating proteins, on the basis of a single band obtained in each case when they were subjected to disc gel electrophoresis (Fig. 2).

Even if the preparations obtained as above were pure, the question may still arise as to whether the substance thus obtained is actually T-H glycoprotein and not some other protein which may be immunologically identical with the T-H. Although no biochemical analyses of this glyco-
protein were done in the present studies, those of others (Fletcher et al. 1970b) suggest that when the glycoprotein obtained as above from human urine is subjected to disc gel electrophoresis and gel chromatography on Sephadex G-200 in the presence of the dissociating agent sodium dodecyl sulphate, each subunit of the glycoprotein thus obtained, appears to be a single polypeptide chain. The molecular weight of each chain was always found to be in the range of 80,000. Similar studies done by Dunstan et al. (1974) on hamster T-H and on rabbit T-H by Marr et al. (1971) confirmed the estimates of Fletcher and colleagues. The molecular weights of a single polypeptide chain of other proteins, on the other hand, were found to be considerably different. For example, the molecular weight of a single polypeptide chain of serum albumin was approximately 68,000 (Dunstan et al. 1974).

All this data clearly suggests that T-H glycoprotein obtained by salt precipitation with sodium chloride is a specific substance, quite different from other proteins found in urine and in plasma. Furthermore, the relative proportions of almost all the amino acids in the hamster, human and rabbit T-H urinary glycoproteins are closely similar (Dunstan et al. 1974). This provides strong evidence for the conclusion that despite the fact that the T-H glycoprotein from hamster urine was isolated by a slightly different technique (i.e., by precipitation with 1.0M NaCl) from that initially used by Tamm and Horsfall in 1952 (i.e., by precipitation with 0.58M NaCl), it is still analogous to the T-H isolated from the urine of other
species.

The localization of T-H glycoprotein in the adult hamster and human kidney

a) By immunofluorescence microscopy

The results of earlier workers using immunofluorescence techniques and studying principally rat and human kidney had been very variable and it would seem highly likely that these inconsistencies were due to variations in the immunological techniques and to the frequent use of unfixed cryostat sections which, in the experience of author, greatly increases the difficulty of histological interpretation. There has also to be considered the possibility of intrinsic fluorescence, a question which appears to have been completely ignored by the previous workers (see below).

Most of the earlier research workers (Friedmann, 1966; McKenzie and McQueen, 1969; Wallace and Nairn, 1971; Lewis et al. 1972) have used freeze dried and fixed or unfixed fresh frozen sections for the localization of T-H glycoprotein. Freeze-dried sections which were first introduced into immunofluorescence microscopy by Marshall (1954) have been preferred by other workers, because they have been shown to give only a low percentage of non-specific staining (Mayerbach, 1959). Similarly, the use of fresh frozen sections, which is by far the commonest histological method for use in the fluorescence antibody technique, has been preferred by others, because as far as the immunological properties are concerned, with this method, proteins (antigens or antibodies) remain substantially unaltered. Conventional paraffin sections, usually prepared
from fixed tissues, on the other hand, have not been used frequently for fluorescence antibody studies, because of the general tendency to cause antigen denaturation.

Although, the use of fresh frozen and freeze-dried sections satisfies one of the prerequisites necessary for immunological reactions, they have certain disadvantages, too. First of all, it is not easy to cut relatively thin sections on the freezing microtome. Secondly, in order to prevent alteration of the integrity and structure of the tissue, freezing and sectioning has to be carried out immediately (which is sometimes not practicable) and the sections stored at low and constant temperature. In the methods mentioned, the material is often used unfixed, and even when fixation is used, it is usually done after sectioning; this leads to the distortion of the tissues, thus making the interpretation of the results very difficult.

In the present studies, both of the above mentioned techniques were tried along with the fixed paraffin sections. The last mentioned method, however, was the one of choice. As described in the results section (page 99), with this method, the intrinsic fluorescence which is sometimes observed in the cells of PCT, was not greater than that found with the first two commonly used techniques (see ante). However, the use of paraffin embedded fixed sections in the author's laboratory is justified on several other grounds. It has been found that T-H glycoprotein has the remarkable property of withstanding the high temperature used in paraffin
embedding. Also, despite the fear that because of the nature of the fixatives used, there might have been some loss of antigenicity, enough reactive T-H glycoprotein was still to be found in situ in precisely those sites where it was observed in the unfixed cryostat sections. This justifies the use of fixed paraffin embedded material in immunofluorescence microscopy.

In the present study, the restriction of specific T-H positive staining to the ALH and the DCT was checked by the parallel examination of neighbouring sections stained by routine histological methods. The morphological features of various portions of the nephron are fairly distinct and the use of phase contrast microscopic attachments with the epifluorescence microscope, greatly facilitated the defining of these features. In addition to differences in tubular size and the shape and size of its epithelial cells, features such as the prominent brush border of the PCT cells also helped in the identification of individual portions of the nephron.

The results of the present study done by the indirect immuno-fluorescent technique shows that T-H glycoprotein is present in the hamster and human kidney slices only in the cells of ALH and DCT. The fluorescence in these parts of the nephron was diffuse and this made the precise cellular localization of the fluorescence difficult to interpret. This diffuseness of the staining was partly a consequence of the thickness of the paraffin sections (5-7 μm) and particularly because of the relatively low resolving power of the light microscope.
T-H glycoprotein was absent in the cells of PCT, a finding which confirms the results of most of the recent workers (on Human kidney, Friedmann, 1966; McKenzie and McQueen, 1969; Pollak and Arbel, 1969; on Human, rat and guinea-pig kidney, Schenk et al. 1971; Wallace and Nairn, 1971 and on cat, mouse, goat and rabbit kidney, Wallace and Nairn, 1971). As explained before, it is possible that some of the earlier results (on human kidney, Keutal, 1964 and on rabbit, Cornelius et al. 1965) suggesting that this glycoprotein occurs within the cells of PCT were due to intrinsic-fluorescence, which has been found to be quite marked in the hamster, especially in unfixed and untreated cryostat sections (Sikri et al. 1979). It is not clear, however, from the published work of previous workers, whether they have in fact, tested for intrinsic fluorescence. A very slight amount of dull green fluorescence in the PCT cells and in the cells of the glomeruli, sometimes seen in fixed paraffin sections which had received the full antibody treatment was considered to be non-specific because similar staining was also found even when the anti T-H antibodies were omitted in control tests. Likewise, fixed but otherwise untreated sections mounted in PBSA sometimes gave similar results.

The presence of T-H glycoprotein in the cells of ALH and DCT has also been reported by others (Friedmann, 1966; McKenzie and McQueen, 1969; Pollak and Arbel, 1969; Schenk et al. 1971; Wallace and Nairn, 1971, Lewis et al. 1972 and Hoyer et al. 1974). In contrast to the results obtained in the present studies (Sikri et al. 1979, Foster
et al. 1979), intense staining in the MD segment has been mentioned by some authors (McKenzie and McQueen, 1969; Schenk et al. 1971 and Hoyer et al. 1974), but it is by no means clear from their published work, whether they refer specifically to the MD cells or to that region of the tubule of which MD is a part. In fact, it is only Wallace and Nairn (1971) who specifically refer to the MD cells, claiming that in man these cells fluoresce in a similar manner to that of the rest of DCT and therefore, contain T-H glycoprotein. Even the claim made by these latter workers requires reconsideration, since an examination of the photograph shown in support of their assertion, does not seem to possess the morphological characteristics of a typical MD. The results of the present study on the hamster kidney, however, show that in those sections where the cells of the MD were clearly identified, they do not fluoresce and therefore, lack T-H glycoprotein.

The cells of the CD of the hamster kidney were also found to lack T-H specific fluorescence, although, this part of the nephron of the human kidney has been reported to contain it (Hermann, 1963; Friedmann, 1966 and Pollak and Arbel, 1969).

The specific presence of T-H glycoprotein in the cells of ALH and DCT has also been confirmed by the immunofluorescence studies on the human kidney. Unlike the claims made by McKenzie and McQueen (1969) and Wallace and Nairn (1971) referred to earlier, T-H positive fluorescence in the cells of the human MD was never found in the present
Although the reasons for the contradictory results obtained are not clear, a possible explanation may be the difficulties which may sometimes arise in interpreting the histology of unfixed cryostat sections, as used by these authors.

It should again be stressed that in the present investigations on hamster and human kidneys, formal calcium chloride/or neutral formalin/or PLP fixation preceded the application of the appropriate immunological reagents. Earlier experiments done on the hamster showed a good correspondence between the results obtained from the study of the best sections from unfixed cryostat material as compared with the fixed material. The fixed material, however, gave a far clearer picture and therefore justifies its use. Furthermore, the use of epifluorescence combined with phase contrast microscopy enabled the histological identification of a fluorescing object to be made with a high degree of certainty.

Another important point which is worth mentioning is that in the present investigations, the indirect 'sandwich' method was used for the localization of T-H glycoprotein. This is in contrast to the work of most of the other workers (Friedmann, 1966; McKenzie and McQueen, 1969; Hoyer et al. 1974; Masuda et al. 1975 and Kirchner and Bichler, 1976) who have used the direct method. The main advantage of the indirect method, which was devised by Weller and Coons (1954) is that it is much more sensitive than the direct one. The sensitivity has been found to be as high as ten times that of direct method (Coons, 1956)
or even up to thirty times as high (Karlsson and Kudynowsky, 1975). This extra sensitivity is mainly attributed to the additional combining sites which are made available by the antibody molecules of the middle layer acting as antigen (in this case: anti T-H antibodies) for the fluorescent antiglobulin (fluorescein-labelled sheep anti-rabbit globulin). In brief, the extra sensitivity provided by the use of the indirect 'sandwich' method is sufficient enough to make up any loss which may occur during the fixation procedure. Furthermore, by utilizing the indirect method, the problem of possible denaturation and loss of antigen (anti-T-H antibodies) during labelling is completely circumvented by employing unlabelled antigen as the primary reagent in the first layer.

Some interesting observations were made by comparing the effect of various fixatives and fixation techniques on the immunofluorescent localization of T-H glycoprotein in the hamster kidney. Although, regardless of the fixative used, the glycoprotein was found to be associated with the ALH and DCT, there was some difference in the distribution of this glycoprotein within the cells of the tubules concerned. For example, when kidneys were fixed in PLP or formal calcium chloride, brilliant fluorescence was obtained, both on the luminal border as well as in the interior of the cells. In paraformaldehyde-glutaraldehyde fixed kidneys, on the other hand, while the luminal border was still fluorescent, almost negligible staining was observed inside the cells. Contrary to earlier reports (Wallace and Nairn, 1971), the duration of fixation in
formalin does not appear to have any noticeable effect on the amount of fluorescence exhibited by the T-H positive tubules. Thus, fixation of kidneys in formal calcium chloride for up to 24 hours neither enhanced nor reduced fluorescent staining.

b) by light and electron microscopical immuno-peroxidase techniques

The next step in the present investigations was to study the localization of T-H glycoprotein in the hamster kidney by immunoperoxidase techniques, both at the light microscopical and electron microscopical levels. Horseradish peroxidase, as an immunological marker has not been used before at the light microscopical level (and indeed electron microscopical level, see later) for the localization of this glycoprotein and the results obtained by the use of this technique form an important part of the present work.

As already stated, most of our knowledge about the localization of T-H glycoprotein is based on the observations of earlier workers, recorded by immunofluorescence studies. This method, however, possesses several inherent disadvantages. The final reaction product is impermanent and fluorescence fades within a very short time of preparation. Furthermore, the immunofluorescence antibody method is inapplicable to electron microscopy. Therefore, the precise intracellular localization of an antigen cannot be demonstrated by this technique.

Most of the above mentioned problems have been overcome by the use of peroxidase labelled antibody techniques (Nakane and Pierce, 1966; Avrameas, 1972) and by the use
of unlabelled peroxidase anti-peroxidase complex (Burn, 1975). Both, the sensitivity and specificity of peroxidase antibody conjugates are now well established (Nakane and Pierce, 1966; Benson and Cohen, 1970; Dorling et al., 1971; Petts and Roitt, 1971 and Seligman et al., 1973).

The use of peroxidase labelled antibodies as an alternative to fluorescence microscopy can be justified by the several clear advantages which this method possesses. First of all, preparations are permanent and can be easily examined with the light microscope. Secondly, sections stained by this technique can be readily counterstained with routine histological stains.

The endogenous peroxidase activity of red blood cells and granulocytes which sometimes present difficulty in the interpretation of results has now largely been overcome (Straus, 1972; Streefkerk, 1972; Taylor and Burn, 1974). In the present study, methanol and H₂O₂ treatment (Streefkerk, 1972) was used for blocking the endogenous peroxidase of blood cells present in the renal capillaries (especially when immersion fixed sections were used). Similarly, the problem of background staining which is sometimes encountered when peroxidase conjugates bind non-specifically on to basically charged tissue components has been successfully overcome by the treatment of kidney sections with the undiluted normal sheep serum prior to the application of antibodies.

The observations made by the immunofluorescence technique on hamster and human kidneys were confirmed by the data obtained with immunoperoxidase methods at the
light microscopical level.

The results obtained with the technique which finally involves treatment of sections with peroxidase labelled sheep anti-rabbit IgG, prepared according to the method described by Avrameas and Ternynck (1971, see results section) were unsatisfactory. Not enough brown reaction product was deposited in the reacting tubules to help in the identification of T-H glycoprotein containing cells with any degree of certainty. Satisfactory results were, however, obtained when the above mentioned conjugate was replaced by the one prepared according to the method of Nakane and Kawaoi (1974), and also by the unlabelled antibody enzyme method of Sternberger (Burn et al. 1974).

In general, the results were in accord with those obtained by the fluorescence technique already referred to. The resulting brown reaction product was observed only in the ALH and DCT. The MD cells were again shown to be free of the glycoprotein.

In formal calcium chloride fixed and paraffin embedded hamster kidney sections, a diffuse overall staining in the form of a dark brown precipitate was observed in the cells of ALH and DCT. The staining of these cells in the paraformaldehyde-glutaraldehyde fixed material, on the other hand, was selective. While the luminal border of these tubules stained heavily for T-H, a reaction inside the cells was only occasionally observed. Similarly, in the PLP fixed kidney sections, the luminal border was once again shown to contain T-H glycoprotein. The reaction in the cell interior
was however restricted to the basal plasma membrane (this has been confirmed by electron microscopical observations, see later).

Some of the problems relating to the penetration of antibodies into the cells are well demonstrated in Figs. 16 & 18. The photograph showing overall staining of the cells of DCT (Fig. 16) is of a formal calcium chloride fixed 5 μm paraffin section of the hamster kidney which was subsequently treated with the immunological reagents. It should be compared with the result depicted in Fig. 18 which represents a 1 μm section cut from a 30-40 μm cryostat section which had been fixed in paraformaldehyde-glutaraldehyde and treated with the immunoperoxidase procedure before embedding in araldite. In the latter type of experiments, staining of the glycoprotein was restricted to the luminal border of the cells, a result which could probably also be related to the method of fixation (i.e., immersion or perfusion, see Figs. 19-21) or to the nature of the fixative.

Further important information about the localization of T-H glycoprotein in the hamster kidney was obtained from studies made with immuno-electron microscopic techniques. Using several different fixatives and peroxidase-labelled antibody techniques, completely new and interesting observations were made. Providing fixation was done by perfusion with PLP reagent, T-H glycoprotein was seen to be present not only on the luminal surface of the cells of the ALH and DCT, but also on the basal plasma
membrane, including its infoldings. Occasionally, Golgi bodies and endoplasmic reticulum were also found to possess the glycoprotein. It thus seems likely from these results that the plasma membranes of the cells in question are generally associated with the T-H glycoprotein, rather than just the cell surface exposed to the tubular urine. Staining of the basal plasma membranes was not often seen when other fixatives were used. In the paraformaldehyde-glutaraldehyde fixed kidneys, for example, T-H was located on the luminal border of the cells of ALH and DCT, and even then in only those ultrathin sections cut very close to the surface of the thick cryostat sections. Those cut from the deeper regions were almost completely negative. Also, in those sections obtained from near the surface, apart from the luminal border, the reaction was not seen anywhere else. This suggests that the lack of a reaction in the deeper sections was due to a penetration problem in the paraformaldehyde-glutaraldehyde fixed material.

It is a well known fact that the stabilization of antigens in situ without destroying their antigenicity and at the same time retaining relatively good tissue and cellular morphology, is a common problem in immunocytochemistry. The majority of conventional fixatives (including paraformaldehyde-glutaraldehyde) interact with proteins (Kraehenbuhl and Jamieson, 1974) and thus, often denature protein antigens. In the present work, the problem of possible denaturation of T-H glycoprotein and the penetration of antibody into thick sections was easily overcome by the use of PLP fixative. Theoretically, the
carbohydrate moieties in tissue are oxidised by the periodate present in this fixative to form aldehyde groups. Lysine, a divalent amine, then cross links the carbohydrate containing molecules (in this case T-H glycoprotein) by reacting with aldehyde groups (McLean and Nakane, 1974), thus greatly reducing both the loss and denaturation of the glycoprotein.

Another interesting point was noted with immuno-electron microscopy. T-H glycoprotein in the hamster kidney was found to be present along the cells of DCT as far as its junction with the adjoining CD. Indeed, the junction of the DCT with the CD acted as a dividing line between the cells which possess the glycoprotein and those which do not.

Also, once again, T-H was found to be absent in the cells of MD, whose luminal border as well as lateral and basal membranes together with their folds were negative.

The results obtained by the immunoelectron microscopy on the human kidney were similar to those of the hamster kidney, although to date specimens from only two patients have become available. Because, in the hamster kidney excellent results were obtained with the periodate-lysine-paraformaldehyde, therefore, only this fixative was used for the fixation of human kidneys. Although, because of the immersion fixation used, the overall preservation of the tissue was not very good, one could still see that the T-H specific deposits of the diaminobenzidine reaction product was restricted to the cells of ALH and DCT. The luminal border and lateral as well as the basal plasma
membranes possessed the glycoprotein. As in the hamster, no staining was observed in the cells of MD.

Three important features thus emerge from these studies:

1) T-H glycoprotein is localized only in the cells of the ALH and DCT and it extends right up to the junction of the latter with the CD.

2) The glycoprotein is associated with the total plasma membrane system of the cells in question.

3) T-H glycoprotein is absent in the cells of MD.

Role of T-H glycoprotein in the adult mammalian kidney

From the present observations and those of others concerning the high viscosity of T-H glycoprotein solutions (Curtain, 1953; Stevenson, 1968; Stevenson, et al. 1971), it is tempting to postulate the following role for T-H glycoprotein in the normal mammalian kidney.

The ability of T-H glycoprotein to produce highly viscous solutions suggests that the aggregated molecules of this glycoprotein can entrap water molecules in a relatively fixed structure. If it is assumed that a similar entrapment of water molecules may also occur in the glycoprotein whilst the latter is still associated with the cell surface, one can envisage a situation in which a barrier of relatively stationary water molecules occurs within an ordered structure. This situation can then be related to
the physiological events believed to be peculiar to the role of ALH and DCT in the process of urine dilution. Thus, T-H glycoprotein (which is present on the surface of the ALH and DCT) because of its property of forming viscous solutions might be expected to act as a barrier with a greatly reduced permeability to water molecules, yet at the same time still allowing relatively small ions of sodium and/or chloride to pass into the interstitium via the cells of ALH and DCT (Sikri et al. 1979). A selective passage of ions across the basal plasma membrane of these portions of the nephron might also be expected to result from the presence of T-H glycoprotein associated with that side and thus prevent water molecules following the Cl and/or Na ions into the interstitium. In this way T-H glycoprotein molecules could help to conserve the water content of these cells.

It is generally accepted that the hyperosmolarity in the medulla of the kidney results from the passage of chloride ions with its accompanying sodium ions across the single cell layer of the lumen of the ALH, a region of the nephron with relatively high impermeability to water (Rocha and Kokko, 1973; Burg and Green, 1973; Burg and Stoner, 1974; Kokko, 1974; Moffat, 1975). On the basis of what has just been said, it is thus postulated that T-H glycoprotein molecules may be responsible for this effect. The presence of the glycoprotein on the surface of the cells of DCT might be expected to reduce the permeability to water of this region, although in the rat and the human at least, this is believed to be regulated
by anti-diuretic hormone (Pitts, 1974; Harvey, 1974). This has been denied by Clapp and Robinson (1966) and Gross et al. (1975). On the other hand, if the present hypothesis which is based on the presence of T-H glycoprotein in the DCT as well as in the ALH, is correct, then it would seem likely that in all the species studied in the present investigations the cells of DCT along with those of ALH, are relatively impermeable to water. This concept is strengthened by the work of Clapp and Robinson (1966) on dogs, of Bennett et al. (1968) on monkeys and that of Morell (1966) on some strains of rats. All these authors have found that the urinary fluid remains hypotonic throughout the entire length of the ALH and DCT.

The absence of the glycoprotein in the MD cells adds weight to this hypothesis because if the function of these cells is to monitor the composition of the luminal fluid, i.e., to sense the concentration of the Cl and/or Na ions of the urine at that part of the nephron as is generally believed (Latta, 1973; Loragh and Sealy, 1973), it is reasonable to expect their surface to be exposed directly to the tubular urine.

The possibility that T-H glycoprotein is in some way involved in electrolyte and water transport in the kidney has been suggested before (Lewis et al. 1972; Schwartz et al. 1973), although no specific hypothesis was put forward. It is worth remembering here that these authors, while working on the human kidney were unaware of the fact that (1) T-H glycoprotein is absent in the cells of MD and (2) that this glycoprotein is associated with the
plasma membranes of the cells of ALH and DCT.

The influence of adrenal cortical hormones on the excretory function of the mammalian kidney is well recognised and it stems from the two fundamental papers published by Loeb and associates in 1932 and 1933. Their interest however, lay mainly in the decrease in sodium content of the blood serum in patients suffering from Addison's disease. From their studies it was concluded that the loss of sodium in these patients was because of adrenal insufficiency and that the adrenals normally exert a regulatory effect on sodium metabolism by modulating kidney function.

Since its first discovery by Loeb and co-workers, intensive work has been done directed towards the elucidation of the role of adrenal hormones in regulating the renal transport mechanism. Thus, it has now been established that while mineral-corticosteroids such as aldosterone influence the electrolyte transport, notably sodium and/or chloride reabsorption; gluco-corticosteroids (especially cortisol) on the other hand, are said to regulate the intermediary metabolism of glucose, as well as the permeability to water of the distal tubular (ALH and DCT) membranes (Kleeman et al. 1958, 1960 & 1964, Cutler et al. 1962; Hierholzer and Lange, 1974).

Bilateral adrenalectomy, since its first introduction by Brown-Sequard in 1856 has been used extensively as an experimental tool for studying adrenal insufficiency along with the specific effects of hormone substitution. As the relative impermeability of ALH and DCT to water is
believed to be controlled, at least in part, by the adrenal cortical hormones, and as T-H glycoprotein has been found to be present only in ALH and DCT (the so-called urine diluting segment), the possible involvement of these hormones in the production and maintenance of T-H has therefore been investigated. Thus, pilot experiments were done on hamsters involving bilateral adrenalectomy (Alexander et al. 1979). Only fluorescent antibody studies have so far been done on these animals, but the results obtained strongly suggest a marked reduction of T-H glycoprotein in both ALH and DCT. Thus, a varying degree of disappearance of T-H glycoprotein which initially started in the DCT (in 4 day adrenalectomized animals) and later extended to the ALH (in 8 day adrenalectomized animals) was observed. These changes were frequently accompanied by the appearance of brilliantly fluorescing casts in the tubular lumen. Sham-operated animals were comparable with unoperated normals. Also, an ultra-structural examination of the kidneys of two of the adrenalectomized animals revealed normal looking organelles (i.e., mitochondria, Golgi body, etc.) suggesting that there had not been any generalised gross pathological changes. The light and electron microscopic studies of Wiederholt et al., (1968) on the kidneys of adrenalectomized rats confirms the above observations.

One of the major defects which develop in adrenal insufficiency is a disturbance of water metabolism. Apparently, in the absence of corticosteroids the organism is unable to excrete its normal water load. This has
been found to be true in both Addisonian patients as well as in adrenalectomized animals such as dogs, cats, rats and mice (Gaunt, 1951; Gaunt and Chart, 1962; Stolte et al. 1968 and Senft, 1969). The defect can be easily demonstrated when adrenalectomized animals are subjected to an exogenous water load, which they are unable to excrete within a normal time period. The impairment of the water excretion capacity develops gradually after adrenalectomy and in rats reaches its full severity after approximately one week (Stolte et al. 1968).

The inability of adrenalectomized animals to excrete a normal water load has been explained by the demonstration of an increased permeability to water of ALH and DCT epithelium (Stolte et al. 1968). According to the above authors who have tested this hypothesis in free-flow microperfusion experiments, the rate of transtubular outflow of water from a hypotonic perfusate injected into functionally isolated segments of ALH and DCT was found to be enhanced in adrenalectomized dogs. Because T-H glycoprotein appears to be confined only to ALH and DCT (that part of the nephron responsible for the process of urine dilution) and because it disappears almost completely after adrenalectomy, it suggests that the formation of an impermeable barrier to water may be related to the presence of T-H glycoprotein. It must, however, be emphasized that the present work is at the moment too incomplete to permit a more definite statement than this.

In evaluating changes caused by total adrenalectomy it should be remembered that both adrenal cortex and
medulla are excised which leads to significant changes in the electrolyte and water content (Crabbe and Nichols, 1960; Kessler et al. 1964; Mariani and Malvin, 1965) of the renal tissue. Therefore, a change in renal function following surgical removal of the adrenals can be related to lack of adrenal hormones, only if appropriate hormone replacement therapy reverses the change. It is a well known fact that the defect in renal water excretion capacity which develops in adrenal insufficiency can be reversed by the application of glucocorticosteroids. Therefore, it will be necessary that in conjunction with the replacement therapy, a comparison of the daily urinary output of T-H, water and sodium as between normal control animals and adrenalectomized animals will have to be made.

Assuming that the hypothesis relating to the function of T-H holds, then the absence of T-H glycoprotein in the cells of CD, can be explained by the fact that their permeability is controlled by a different cellular mechanism (i.e. by the antidiuretic (ADH) hormone of the posterior pituitary). This is not surprising, because of the different embryological origin of this segment. Collecting ducts are formed as a result of the out-growth and repeated bifurcation of the ureteric bud, which itself is derived from the mesonephric duct (Huber, 1910). In contrast, ALH and DCT along with other parts of the nephron originate from the growth and differentiation of the metanephric blastema (Huber, 1910). Thus, the varying permeability of the CD in the absence of T-H glycoprotein may be related not only to the histological differences of this segment but also to its different embryological origin.
Ontogenic Development of T-H Glycoprotein in the Hamster Kidney and the possible Physiological Significance of its presence in the Foetal Kidney

T-H glycoprotein was first demonstrated by immunofluorescence and immunoelectron microscopical techniques in the distal tubules (by distal tubules is meant that part of the foetal nephron which will ultimately differentiate into ALH and DCT) of 12 days foetal hamster kidneys. Initially, the glycoprotein was observed only in the medulla but with increasing foetal age up to full term, positive staining began to appear in the juxtamedullary as well as cortical regions. It was after birth however, that the positively staining regions could be confidently identified as ALH and DCT. At no stage was there any evidence for the association of T-H glycoprotein with the cells of PCT, thin part of Henle's loop or CD.

By immunofluorescence and by immunoelectron microscopy, it was shown that during early stages of foetal kidney development (12 and 13 days post conception), the greatest amount of T-H was present only on the surface of reacting tubules. An adult-type pattern of localization was however, clearly observed in the kidneys of hamsters just before birth. Thus, in the late foetal (15 days post conception) kidneys, T-H was found to be associated not only with the luminal surface of the tubules concerned but also with the lateral and basal infoldings of the plasma membrane. Cells of the MD were first identified in the 3 days neonatal kidneys and they were negative for T-H.

These studies are in agreement with those of Wallace
and Nairn (1971) who, by using immunofluorescence microscopy, first observed T-H positive staining in the kidney of an 8-week human foetus. Similarly, and likewise employing immunofluorescence techniques, Hoyer et al. (1974) demonstrated this glycoprotein within the cells of distal renal tubules of foetal rats 2 days prior to birth and also in a human foetal kidney of 14-week gestation. Lewis et al. (1972) on the other hand, could not find any T-H positive staining in the foetal rat kidney and therefore suggested that the formation of T-H glycoprotein in the mammalian kidney is associated with the exposure of animal to an extrauterine environment. The reason why the results obtained in the present studies (along with those of Wallace and Nairn, 1971 and Hoyer et al. 1974) are different from those of Lewis et al. (1972) is not clear, although as suggested by Hoyer and colleagues, differences in the potency of antisera and in methods of tissue preparation and staining may have played important roles.

There is very little data available on the development and differentiation of the foetal kidney tubules in mammals and even this is limited to a very few species. The time periods of maturation of various processes concerned in kidney function (i.e. active sodium and chloride transport, urine dilution/concentration ability etc. etc.) at full term also varies from species to species. In the rat, for example, it has been found that the kidneys of newborns are functionally immature and have little ability to dilute urine (Oh et al. 1965). Here it is interesting to note that in this species, T-H glycoprotein was first observed
only one to two days before birth and even then in only small traces (Wallace and Nairn, 1971). In man, on the other hand, the metanephric tubules begin to excrete dilute urine as early as 9 weeks after gestation (Gersh, 1937) i.e. at a time when T-H is first observed (Wallace and Nairn, 1971; Hoyer et al. 1974). The work done by McCance and Stanier (1960) on rabbits and pigs supports the observations of Gersh. These authors found that in the two species mentioned, the foetal kidneys are capable of forming a fluid which resembles adult urine.

There are no physiological data available on the foetal hamster kidney. However, if the present hypothesis is correct and T-H glycoprotein forms a relatively impermeable barrier to water then the presence of this glycoprotein in the distal tubules of hamster's foetal kidney will strengthen the belief that these kidneys are functionally active and that the chloride and/or sodium ion reabsorption and urine concentration/dilution mechanism has already started, at least to a limited extent.
The Ultrastructure of the MD of the Hamster Kidney and the possible Physiological Significance of the lack of T-H Glycoprotein

The results of the present investigations on the hamster kidney (Foster et al. 1979) show, as have those of others on different species, that the cells of MD are sufficiently different in ultrastructure to warrant the belief that they are functionally distinct from the cells of the neighbouring DCT and ALH. The following appear to be the most striking characteristics which differentiate MD cells from those of ALH and DCT in the hamster:

1) The mitochondria show predominantly circular and elliptical profiles and are present in the apical as well as the basal cytoplasm and lack the orderly array seen in ALH and the rest of DCT.

2) The complex infoldings of the basal plasma membrane are more randomly orientated and do not project as far into the cytoplasm as in ALH and DCT.

3) The positions of Golgi complexes are variable. They are by no means always infranuclear as has sometimes been claimed for other species.

4) Subsurface vacuolation is more evident than in the ALH and DCT.

5) The presence of sparsely distributed membrane-bounded spherical dark bodies which are not unequivocally lysosomes.
6) T-H glycoprotein associated specifically with the plasma membranes of the cells of ALH and DCT is absent in MD cells.

The so called 'dark' and 'light' cells of Muylder (1945a) observed by others (in rat, mouse and guinea-pig by Bucher and Krastic, 1971, 1973, 1975 and in rat by Hagege and Richet, 1975) were never found in the MD of hamster kidneys.

Although, it is unwise to make any physiological deductions from the morphological data, it is tempting to speculate concerning the possible significance of some of the characteristics listed above. It is a widely held but not conclusively established belief that the cells of MD monitor the sodium and/or chloride ion concentration of the urine passing through the DCT (Latta et al. 1962; Brown et al. 1966; Thurau et al. 1967; Vander, 1967 and Latta, 1973) and in some way, convey this information to the renin-producing cells of the juxtaglomerular apparatus (Tisher et al. 1968). Alternatively, it has been suggested that the cells of MD are sensitive to the rate of urine flow and by a feed back mechanism regulate the glomerular filtration rate (Goormaghtigh, 1945; Hierholzer et al. 1978).

The MD cells of the hamster and other species do not show the structural polarity which appears to characterise those of the rest of the nephron - a polarity that is essentially similar to that of exocrine cells. This itself, suggests a different function for the MD cells but does not
immediately indicate what that function might be.

The polarity of the cells of PCT, ALH and DCT associated as it is with numerous and regularly oriented basally situated mitochondria appears to be correlated with the active transport mechanisms which these cells are thought to possess. In contrast, the more dispersed mitochondria and the shallow and less clearly orientated basal infoldings of the plasma membrane of the cells of MD suggest that active transport is not a prime function of this region. This, coupled with the very numerous sub-surface vacuoles (apparently of pinocytotic origin), admits the possibility of some sort of urine sampling activity. Whether the variable positions of Golgi complexes in the hamster MD can be correlated with such a monitoring function is by no means clear - in fact it seems unlikely. On the other hand, as has been mentioned earlier, a permanently infranuclear position of the Golgi apparatus (i.e., a reversal of polarity) has been claimed for a number of other species and this has been linked to the notion that MD cells may have a secretory activity directed towards the extraglomerular mesangium and the renin-containing juxtaglomerular cells. If this is indeed the case then, the variable position of this organelle in the hamster suggests that, at any given time, only a proportion of the MD cells (i.e. only those cells of the MD with infranuclear Golgi) are functioning in this manner.

In several species (including the hamster) where the presence of membrane-bounded dark granules, with a sparse
and predominantly basal distribution have been observed, it has been suggested that these might represent a secretion product (Tisher et al. 1968; Latta, 1973). In the hamster, however, it has not been possible to differentiate unequivocally between these bodies and lysosomes.

Perhaps the most significant finding, has been the total absence of T-H glycoprotein in the MD cells of the hamster and human kidney. As has been mentioned earlier, and as is briefly summarised here, this protein is associated with the plasma membranes of the cells of the ALH and all other cells of DCT. It is absent in the cells of PCT, thin part of Henle's loop and CD. In view of this specific distribution, an hypothesis has been advanced (Foster et al. 1979; Sikri et al. 1979) which suggests that there is likely to be a correlation between the presence of T-H glycoprotein and the special function of this part of the nephron, i.e., the active transport of chloride and sodium ions outwards into the interstitium (Pitts, 1974). Since this ionic movement is not accompanied by a corresponding water flow, it has been suggested that T-H glycoprotein (presumably because of its particular molecular structure and also because of the property of its molecules to form highly viscous solutions) impedes the flow of water molecules from the tubular urine into and hence out of the cells. In the light of this supposition, it would seem that the absence of this glycoprotein in MD cells becomes of particular significance - it would permit direct sampling of the urinary fluid and thus facilitate the monitoring activity that many investigators believe these cells to possess.
ADDENDUM
Localization of T-H Glycoprotein in the Normal and Adrenalectomized Rat Kidney

While this thesis was being written, some work has also been done on rats, in order to extend the interesting observations recorded on the localization of T-H glycoprotein in the normal adult and adrenalectomized hamsters and the adult human kidneys.

Both immunofluorescence and immunoelectron microscopical techniques were used for the localization of this glycoprotein in the rat kidney. In general, the techniques used were similar to those described before, for the hamster and human.

MATERIALS AND METHODS

1) Animals

A total of 19 adult Wistar and 3 Sprague-Dawley rats were used. Of the former, 10 were used for normal immunohistochemical studies and on the remainder, adrenalectomies were carried out. All 3 Sprague-Dawley rats, on the other hand, were utilized in the adrenalectomy experiments.

2) Rat T-H glycoprotein

T-H glycoprotein was isolated from the rat urine by multiple precipitation with 0.58M sodium chloride (Tamm and Horsfall, 1952). It showed one band on electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate (Fig. 2c).
3) Rabbit (anti rat T-H) antibody

Antibodies to rat T-H glycoprotein were raised in rabbits by injecting 1 ml of an emulsion containing equal volumes of glycoprotein (1 mg/ml in water) and complete Freund's adjuvant. Two further injections were given at fortnightly intervals and 10 days after the last injection the blood was drawn from the ear vein and collected in sterile bottles. Rabbit-(anti rat T-H glycoprotein) IgG was isolated from the antiserum by the procedure of Sober and Peterson (1958). The samples containing IgG were collected, dialysed against water and after dividing the solution into small portions, it was immediately frozen.

Procedures for the isolation of IgG from sheep(anti-rabbit globulin) serum and the preparation of conjugates of HRPO with sheep-(anti rabbit globulin) IgG were similar to those described before (see pages 43 - 46)

4) Adrenalectomy

As mentioned earlier, total bilateral adrenalectomies were performed on 7 adult Wistar and 3 Sprague-Dawley rats. Sham operations were done on 2 Wistar rats.

The method used for conducting adrenalectomy was similar to that applied to the hamster (see page 66). Animals were fed on normal pellet food and 0.9% saline was provided for drinking.

Wistar rats were killed on 3, 4, 5, 6, 7, 15 and 20 days post-adrenalectomy, while the sham operated animals were sacrificed on the 7th and 15th day. Similarly,
kidneys from Sprague-Dawley rats were removed 15, 18 and 20 days after adrenalectomy. The reason why these particular dates were chosen for sacrifice of the latter animals was because Dr. G. V. Groom (personal communication), using a specially sensitive assay technique has found that, in this strain, the level of circulating cortico-sterone virtually dropped to zero after approximately 2 weeks post-adrenalectomy. This therefore, seemed an appropriate time at which to investigate the effect of adrenalectomy on the production of T-H glycoprotein.

5) Fixation and Processing

The adrenal glands and in one case, accessory adrenal nodules (adrenal rests, see later), were fixed by immersion in formal calcium chloride and processed for light microscopy.

The kidneys were fixed by vascular perfusion at a pressure of 160 mm mercury. For immuno-fluorescence microscopy, kidneys were fixed in the same way as in the hamster, except that the fixative used was always formal-calcium chloride. Similarly, for immunoelectron microscopy, kidneys were fixed in PLP and processed as described before for the hamster.

The cryostat sections (20-30 μm thick) were cut from different levels of the cortex and medulla and after washing in PBSA, procedure† (used for the immunoperoxidase staining of the hamster and human kidney sections, see page 60 ) was followed. In this case
rabbit anti hamster/human T-H antibodies, were however, replaced by the corresponding antibodies of the rat.

RESULTS AND DISCUSSION

1) The Localization of T-H Glycoprotein in the normal Wistar Rat

Immunofluorescent microscopy showed that as in the hamster and human, T-H in the rat kidney was clearly localized in the ALH (Fig. 82) and in the DCT (Fig. 83) only. The staining of the cells was diffuse and both the luminal surface as well as the cell interior (except the nucleus) fluoresced brilliantly.

The most interesting finding in the rat kidney was that two types of MD were observed - those which completely lacked T-H glycoprotein (as in the hamster and human, Fig 84) and those which possessed some degree of T-H positive luminal staining (Fig. 85). The latter type appeared to be much more frequent. The MD of either type were scattered randomly throughout the cortex.

The results of immunoelectron microscopy confirmed the observations recorded by immunofluorescence microscopy. It was observed that although T-H glycoprotein is invariably associated with the cells of ALH (Fig. 86) and DCT (Fig. 87), it is always absent in the PCT, thin limb of loop of Henle and likewise in the CD and glomerulus.

The examination of ultrathin sections taken from the kidneys fixed in PLP reagent, showed the presence of this glycoprotein not only on the luminal side of the cells of ALH and DCT but also in the lateral as well as basal plasma
membranes, including the infoldings of the latter (Figs. 88, 87).

As far as the localization of T-H in the MD is concerned, once again, two different types were observed - those which contained the glycoprotein and those which didn't. The first type of MD were seen to possess T-H glycoprotein only on the luminal border of its cells (Figs. 88, 89). The lateral and basal membranes including their invaginations were always negative. No staining was observed in the cells of the second type of MD (Fig. 90).

Thus it is seen that the localization of T-H in the ALH and DCT of the rat kidney is precisely similar to that observed in the hamster and in man. It is therefore reasonable to assume that the function of T-H glycoprotein here, is similar to that in the hamster and human already mentioned, in that it imparts a considerable degree of impermeability to water.

The presence of T-H glycoprotein on the luminal surface of some of the MD in the rat is somewhat surprising, as this is in contrast to the situation in the hamster and human where all the MD observed were found to be T-H free. If the present hypothesis is correct then it would seem that at any given time, in this species, only a portion of MD i.e. those which completely lack the glycoprotein are engaged in the monitoring operation. The possibility cannot, however, be ruled out that the reaction observed on the surface of some of the MD is not indeed T-H glycoprotein but some other protein which is immunologically identical with T-H.
The Localization of T-H Glycoprotein in the Kidneys of Adrenalectomized Wistar and Sprague-Dawley Rats

In the kidneys of Wistar rat killed 3 days post-adrenalectomy, fluorescence microscopy showed some reduction of T-H in the cells of most of the DCT (Figs. 91). The effect was however, limited to the DCT of the outer cortex only. On this day, the cell interior of the majority of affected tubules, appeared to have lost their T-H specific fluorescent staining while their luminal border still fluoresced. The lumen of these tubules was also found to be occasionally blocked with T-H positive casts (Fig. 92).

The number of DCT showing the effect of adrenalectomy and the extent of reduction of T-H was much less in the animals of both strains killed on 7, 15, 18 and 20 days post-adrenalectomy. In the 15 days adrenalectomized rats for example, apart from some of the DCT in the outer cortex, the rest of the tubular system still possessed T-H positive staining (Fig. 93) which was in no way different from that of the normal control rats. There appears to be no effect of adrenalectomy on the cells of the MD. As in the normal controls, the luminal border of most of the MD was found to contain the glycoprotein (Fig. 94), while others were totally negative.

The results obtained with immunoelectron microscopy were similar to those obtained by immunofluorescence microscopy, described above. In Fig. 95, which has been taken from the outer cortex of a 3 days adrenalectomized
Wistar rat kidney, a substantial reduction in the amount of T-H can be seen in the lateral plasma membranes and the invaginations of the basal plasma membrane of a cell of the DCT. The luminal surface of this cell, on the other hand, still contains the dark reaction product. This should be compared with the DCT shown in Fig. 96 which has been taken from the kidney section of a 20 days adrenalectomized rat. In the latter, only a small reduction of T-H is seen in the membrane system, suggesting that the cells had again started producing the glycoprotein. Thus, contrary to the results obtained in hamsters, where with the increasing time after adrenalectomy there was a gradual increase in the number of the affected tubules; the loss of T-H in rats was restricted only to the DCT of the outer cortex and even here the effect of adrenalectomy was transitory.

It has been shown by several workers that in some strains of rats, accessory adrenal cortical tissue can be located near the adrenals themselves (Waring and Scott, 1937) where they form nodules or in the perirenal fat (Dribben and Wolfe, 1947). According to Russfield (1967), these nodules or the so called 'adrenal rests' resemble the normal cortical tissue in that they possess the three usual cortical zones but lack medullary cells. Although their origin and physiological role in the normal animals is not clear (Russfield, loc. cit.), they have been found to become active during malfunctioning of the adrenal glands (Hummel, 1958) and become of significance in experiments when the adrenal glands are removed (Chester Jones, 1957).
On examination, similar accessory nodules were also found in one of the adrenalectomized Sprague-Dawley rats used in the present studies. Haematoxylin and eosin stained light microscopical preparations showed that these nodules are covered by a capsule followed by a thin outer layer of zona glomerulosa and a thick inner layer, the zona fasciculata (Fig. 97) and thus, completely lack the zona reticularis and the medulla found in the adrenal glands of normal animals (Fig. 98).

If the present hypothesis (see pages 123-27) which is based on the assumption that the production and maintenance of T-H glycoprotein is under the influence of adrenal cortical hormones is correct, then the apparent lack of any clear cut response to adrenalectomy (such as was observed in the hamster) in rats, could be accounted for by the presence of accessory adrenal tissue. Furthermore, since these results are based on the study of a limited number of animals, more experiments need to be performed before coming to a definite conclusion.
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D.7. Some observations on the ultrastructure of the macula densa of the distal convoluted tubule of the kidney of the Syrian hamster. By C. L. Foster and K. Sikri (introduced by A. D. Hoyes). Department of Cellular Biology, St. Mary's Hospital Medical School, London

During the course of a study on the localization of Tamm–Horsfall glycoprotein within the nephron of the kidney of the Syrian hamster, the opportunity was taken of examining the ultrastructure of the macula densa and the neighbouring region of the distal convoluted tubule.

Interest in this structure stems from the notion that it may constitute a mechanism for monitoring sodium and chloride levels in the urine flowing past it. In an early account, McManus (Quart. J. Med. Sci. 85, 1945), using light microscopical methods, claimed an infranuclear position for the Golgi elements in the cells of the macula densa in the rabbit and the cat, as well as an absence of a basement membrane in this region. This aroused great interest, since it suggested a reversed polarity of the cells with the possibility of some secretory product being transmitted to the renin-producing cells which are in close proximity.

The results of ultrastructural studies on the hamster macula densa in general concur with the recent work of others on the rat, the mouse and the human, and support the view that macula densa cells differ in a number of important respects from those of the rest of the distal convoluted tubule. Among these differences may be mentioned the predominantly circular and elliptical profiles of the mitochondria which are much more widely scattered in the cytoplasm than in the distal convoluted tubule. The basal folds of the plasma membrane, unlike those in the distal convoluted tubule, are restricted to the basal region of the cell, where small numbers of dense membrane-bounded (?secretory) granules are sometimes found. The Golgi complexes, however, were observed in a variety of situations ranging from supranuclear to infranuclear, suggesting that this organelle may not occupy a constant position – even perhaps in the same cell. In this respect, the hamster appears to differ from the mouse, the rat and man – the species on which most of the recent work has been done.
9. The localization of Tamm–Horsfall glycoprotein in the nephrons of the kidney of the Syrian hamster as demonstrated by immunofluorescence and immunoelectron microscopical techniques.

By C. L. Foster, R. D. Marshall*, K. Sikri, F. Bloomfield* and D. Pauline Alexander† (introduced by A. D. Hoyes). **Departments of Cellular Biology, *Chemical Pathology and †Physiology, St Mary’s Hospital Medical School, London**

In 1951 Tamm & Horsfall described a mucoprotein (TH) of high molecular weight in human urine which inhibited haemagglutination induced by myxoviruses. A similar protein is now known to be present as a constituent of normal urine in other mammalian species and there is evidence to suggest that TH is a product of the nephron, from which it is released into the urine. Cultured cells derived from baby hamster kidneys likewise synthesize and release TH into the medium. The normal function of the protein, however, remains obscure.

The precise site of production of TH has been a matter of controversy, but recent work using fluorescent antibody techniques suggests that it is associated with the cells of the ascending limb of Henle’s loop and the distal convoluted tubule in rats and in man.

Following work on the production of TH by baby hamster kidney cells, the adult hamster kidney has now been examined with a view to identifying the intrarenal location of TH. Peroxidase-labelled antibody methods were used at the electron microscope level in addition to fluorescent antibody techniques combined with phase contrast microscopy. The results show a very precise localization of TH in the cells of the ascending limb of Henle’s loop and the distal convoluted tubule, except that in the cells of the macula densa TH is virtually absent—an observation not previously reported. Further, this mucoprotein appears to be associated with the plasma membranes of the cells.

On the basis of these new observations and the interesting fact that TH appears to be restricted to that part of the nephron believed to possess a low permeability to water and involved in the urine dilution process, it is postulated that the absence of TH from the cells of the macula densa permits them to ‘sense’ the constituents of the luminal fluid without significantly changing its composition.

![Fig. 4](image-url)
10. The effects of bilateral adrenalectomy on the Tamm–Horsfall glycoprotein of the nephron of the kidney of the Syrian hamster—some pilot experiments. By D. Pauline Alexander, C. L. Foster* and K. Sikri* (introduced by A. D. Hoyes). Departments of Physiology and Cellular Biology, St Mary’s Hospital Medical School, London

As described in the preceding abstract (Communication 9), the urinary Tamm–Horsfall mucoprotein (TH) first reported by Tamm & Horsfall in 1951 is, in the hamster, associated with the plasma membranes of the cells of the ascending limb of Henle’s loop and the distal convoluted tubule, with the notable exception of the macula densa.

Therefore the localization of TH appears to be confined to that part of the nephron responsible for the process of urine dilution. As this function is at least in part regulated by adrenal cortical hormones, the effect of adrenalectomy on the distribution of TH has been studied.

Total adrenalectomies (which are technically difficult in the hamster) were performed on 8 adult animals and sham operations on 4. Of the former, 6 survived the operation and kidneys were removed at intervals ranging from 4 to 8 days. The sham-operated animals were killed after 8 days. Fluorescent antibody techniques were applied to tissue sections and these were then examined under an epifluorescence system with a phase-contrast attachment.

The results obtained with the sham-operated animals were identical with unoperated controls; the adrenalectomized hamsters, however, (with one exception, where possibly adrenalectomy was incomplete) showed varying degrees of disappearance of TH, initially from the distal convoluted tubule and later from the ascending limb of Henle’s loop. These changes were frequently accompanied by the appearance of brilliantly fluorescing casts. These preliminary observations were discussed and data on urinary sodium levels presented.
THE TAMM-HORSFALL GLYCOPROTEIN: ITS STRUCTURE, METABOLISM AND A HYPOTHESIS FOR ITS ROLE IN RENAL FUNCTION.


There is, in the urine of normal individuals, a glycoprotein which can inhibit in vitro haemagglutination induced by influenza virus and other myxoviruses(1). It is termed Tamm-Horsfall glycoprotein or uromucoid although the latter term has sometimes been used to described protein preparations from urine which are not completely homogeneous.

Examination by immunofluorescence and by immunoperoxidase techniques, with the use of the light microscopy and of the electron microscope, of kidney slices from the hamster has revealed a number of important features. Firstly the glycoprotein is associated with the plasma membrane of the cells of the ascending limb of the loop of Henle and of the distal convoluted tubule only, but with the important exception of the macula densa cells. The type of fixative and the technique for fixation was found to be important in order to demonstrate that the glycoprotein is associated with the basal plasma membrane as well as with the luminal surface of the cells in question. From these data and from other known properties of the Tamm-Horsfall glycoprotein it is suggested that the property of the ascending limb of the loop of Henle of allowing relative impermeability to water molecules but allowing chloride ions with their associated sodium ions to pass is due, in part, to the presence of the glycoprotein. The hypertonicity of the medulla which results from this selective passage of molecules is generally accepted as being necessary for the reabsorption of water from the tubular urine in the collecting ducts under the influence of vasopressin. The absence of the glycoprotein on the maculae densae is suggested as being necessary to allow this region of the tubule to act as a sensor for the concentration of ions in the tubular urine at that point. It should be emphasised that the hypothesis is supported by the presence of the glycoprotein on the basal plasma membrane.

Tamm-Horsfall glycoprotein has a sub-unit molecular weight of about 80,000, of which about 25% is carbohydrate. Digestion of the whole glycoprotein or of the asialo-derivative with the use of pronase leads to extensive cleavage of the peptide bonds in spite of the fact that about one in twelve of the amino acid residues of the polypeptide chain are engaged in intramolecular cystine bridges. Fractionation of the digests of the glycoprotein led to the separation of glycopeptides, the compositions and molecular weights of which suggest that the carbohydrate moieties in the original glycoproteins are predominantly of two sizes. One of these has a molecular weight of the order of 4,000 and the other one of about 2,200.

Biosynthesis of the glycoprotein appears to follow a complex process. Homogenisation of hamster kidney was followed by subcellular
fractionation under appropriate conditions to yield enriched fractions of rough and smooth endoplasmic reticulum, Golgi apparatus and plasma membrane. Tamm-Horsfall glycoprotein was isolated from these fractions with the use of affinity chromatography in which anti-(Tamm-Horsfall glycoprotein)-IgG linked to Sepharose 4B was used as the support phase. The sugar compositions of the glycoproteins isolated from these fractions, expressed as residues/80,000 g were:

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<th>Sugar</th>
<th>Composition of T-H glycoprotein isolated from</th>
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<td>Fuc</td>
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<td>Gal</td>
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<tr>
<td>GalNAc</td>
<td>14</td>
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<tr>
<td>GlcNAc</td>
<td>14</td>
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<tr>
<td>NeuNAc</td>
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These data suggest that fucose is added to a large degree in the Golgi apparatus, and sialic acid at the plasma membrane of the cells. Some of the N-acetylglucosamine residues are added at the rough endoplasmic reticulum as are a considerable number of mannose residues. But a full interpretation of the data must await elucidation of the structures of the glycoproteins isolated at the various stages of its biosynthesis.


Localization by Immunofluorescence and by Light- and Electron-Microscopic Immunoperoxidase Techniques of Tamm–Horsfall Glycoprotein in Adult Hamster Kidney

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1. Tamm–Horsfall glycoprotein was isolated from hamster urine and antiserum against it was produced in rabbits. Immunoglobulin G was isolated from the antiserum. 2. Indirect methods of immunofluorescence staining were applied to kidney sections previously fixed by both perfusion and immersion methods. Tamm–Horsfall glycoprotein was identified associated with only the cells of the ascending limb of the loop of Henle and the distal convoluted tubule. Maculae densae were free of the glycoprotein. 3. Indirect immunoperoxidase procedures with light microscopy were applied to kidney sections. The results extended those found by immunofluorescence by showing that the glycoprotein is largely associated with the plasma membrane of the cells. Macula densa cells were shown to be free of the glycoprotein, although the luminal surface of the remaining cells in the transverse section of the nephron at that region was shown to contain it. 4. A variety of immunoelectron-microscopic techniques were applied to sections previously fixed in a number of ways. Providing periodate/lysine/paraformaldehyde was used as the fixative, the glycoprotein was often seen to be present not only on the luminal surface of the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule, but also on the basal plasma membrane, including the infoldings. 5. It is generally accepted that the hyperosmolarity in the medulla of the kidney results from passage of Cl− ions with their accompanying Na+ ions across the single cell layer of the lumen of the thick ascending limb of the loop of Henle, a region of the nephron with relatively high impermeability to water. We suggest that Tamm–Horsfall glycoprotein operates as a barrier to decrease the passage of water molecules by trapping the latter at the membrane of the cells. Our hypothesis requires the glycoprotein on the basal plasma membrane also.

The Tamm–Horsfall glycoprotein is of considerable interest. It occurs in the urine of a number of species of placental mammals (Tamm & Horsfall, 1950, 1952; Mia & Cornelius, 1966; Wallace & Nairn, 1971; Dunstan et al., 1974; Masuda et al., 1977), and its main, if not its only, organ of origin is the kidney (Cornelius et al., 1965). The human glycoprotein inhibits haemagglutination induced in vitro by myxoviruses (Tamm & Horsfall, 1950, 1952), but the hamster and rabbit glycoproteins do not (Bloomfield et al., 1977), and the function of the glucoprotein is unknown.

A number of studies have been made by immunofluorescence methods of the localization of Tamm–Horsfall glycoprotein in kidney slices. The results have shown considerable disagreement, and the glycoprotein was reported to be present in the cells of the proximal convoluted tubule (Keutel, 1965; Cornelius et al., 1965), in those of the loops of Henle (Friedmann, 1966; Pollak & Arbel, 1969; Wallace & Nairn, 1971), of the thick ascending limbs of the loops of Henle (McKenzie & McQueen, 1969; Schenk et al., 1971; Hoyer et al., 1974) of that part of the renal tubule in the region of the macula densa (McKenzie & McQueen, 1969; Schenk et al., 1971), of the distal convoluted tubule generally (Friedmann, 1966; McKenzie & McQueen, 1969; Hoyer et al., 1974), of the tubules generally (Masuda et al., 1975) and of the collecting ducts (Herman, 1963; Pollak & Arbel, 1969). The brush border and smooth endoplasmic reticulum of the cells of the proximal convoluted tubule were also suggested to contain the glycoprotein from the results of experiments done with ferritin-labelled antiserum (Pape & Maxfield, 1964).

The discrepancies noted made it desirable to reinvestigate the problem with the use of a number of procedures. Hamster kidneys were fixed by a variety of techniques before localizing the glycoprotein in slices by immunofluorescent methods and by immunoperoxidase techniques with the use of both
light microscopy and electron microscopy. In all the experiments described, the indirect method for the localization of the antigen was used.

Preliminary data have been reported (Foster et al., 1979; Foster & Sikri, 1979).

Experimental

Materials

Horseradish peroxidase (type VI, RZ approx. 3.0) was from Sigma (London) Chemical Co., London S.W.6, U.K., and 3,3'-diaminobenzidine was from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Hamster Tamm–Horsfall glycoprotein

This was isolated from hamster urine by a procedure involving, finally, chromatography on Sepharose 4B (Dunstan et al., 1974). The preparation was freeze-dried. It showed one band on electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate (Fig. 1).

Antiserum

Antiserum to hamster Tamm–Horsfall glycoprotein was raised in New Zealand White rabbits. Injections were made intramuscularly of 1 ml of an emulsion containing equal volumes of glycoprotein (1 mg/ml in water) and complete Freund's adjuvant (Calbiochem, Hereford, U.K.). A total of at least three injections was given at fortnightly intervals. Blood was taken from the ear vein of the rabbits between 7 and 10 days after the last injection and the serum was collected.

Isolation of immunoglobulin antibody

Rabbit anti-(hamster Tamm–Horsfall glycoprotein) immunoglobulin G. This was isolated from the samples of antiserum by the procedure of Sober & Peterson (1958). It was dialysed against water and the solution was freeze-dried. The product was dissolved in the same volume of water as the serum from which it was derived and the solution was stored in small portions at –20°C. For use in the experiments to be described, a 10-fold dilution of the aforementioned solution was made with phosphate-buffered saline (Dulbecco A buffer; Dulbecco & Vogt, 1954).

Fluorescein-labelled sheep anti-(rabbit immunoglobulin) serum. This was bought from Wellcome Laboratory, Pirbright, Surrey, U.K. The freeze-dried material (7.6 mg of protein on the basis of the biuret method; 2.95 mg of antibody protein) was dissolved in 1 ml of Dulbecco A buffer; it was diluted eight times before use.

Sheep anti-(rabbit globulin) immunoglobulin G. This was isolated (Sober & Peterson, 1958) from antiserum purchased from Gibco-Bio-Cult, Paisley PA3 4EP, Scotland, U.K. For some experiments the combined fractions from the DEAE-cellulose column containing immunoglobulin G were dialysed against either Dulbecco A buffer or Tris/saline buffer, pH 7.6 (0.046 M-Tris/0.139 M-NaCl), and diluted 2-fold in the respective buffers. For other experiments the immunoglobulin G obtained from the column was dialysed against water and the solution was freeze-dried.

Preparation of conjugates of horseradish peroxidase with sheep anti-(rabbit globulin) immunoglobulin G. Method 1. Peroxidase (10 mg) was coupled to 5 mg of immunoglobulin G by the procedure of Avrameas & Ternynck (1971) in which glutaraldehyde was used for linking the enzyme to the antibody.

Method 2. Peroxidase (5 mg) was converted into poly-(ε-N-dinitrophenyl)peroxidase before linking of the latter to immunoglobulin G (5 mg) by the method of Nakane & Kawaoi (1974).

Soluble complex of horseradish peroxidase and rabbit anti-(horseradish peroxidase) immunoglobulin (Sternberger et al., 1970) was. This kindly provided by Dr. L. A. Sternberger, Chemical Systems Laboratory, U.S. Army Armament Research and Development Command, Aberdeen Proving Ground, MD, U.S.A.

The solution as supplied contained the equivalent of 0.93 mg of horseradish peroxidase and 2.91 mg of antiperoxidase per ml. For use in the experiments it

Fig. 1. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Marshall & Zamecnik, 1969) of the Tamm–Horsfall glycoprotein
was diluted 40-fold with Tris/HCl buffer, pH 7.6, containing NaCl (0.046 M-Tris, 0.139 M-NaCl), which also contained 1% normal sheep serum. This solution is referred to as the PAP reagent below.

**Fixation of kidneys**

**Immersion fixation.** After intramuscular injection of hamsters with Nembutal (0.2 ml), the kidneys were removed and cut into small pieces, which were immersed for 16 h at 22°C in aqueous formaldehyde solution containing Ca²⁺, described below as formal/Ca²⁺ [1 vol. of commercial formaldehyde solution (40%, w/v) neutralized with phosphate buffer and 1 vol. of 10% CaCl₂ + 8 vol. of water].

The kidneys were washed in running water for 2-4 h and dehydrated [1 h successive immersion in aq. 50%, 70%, and 90% (v/v) ethanol followed by absolute ethanol (several changes)]. They were cleared by immersion in chloroform overnight and finally embedded in paraffin wax.

**Perfusion fixation.** This was carried out with apparatus modified from that of Rossi (1975). Animals were anaesthetized by intramuscular injection of 0.2 ml of Nembutal, and fixed by vascular perfusion for 10 min at 140 mmHg pressure through the abdominal aorta (Maunsbach, 1966). A variety of fixatives was used as follows: (a) solutions of paraformaldehyde (1% or 2%, w/v) containing glutaraldehyde (0.05-0.5%, v/v) in 0.2 M-sodium cacodylate buffer, pH 7.4; (b) the sodium periodate/lysine/paraformaldehyde fixative of McLean & Nakane (1974), in which the paraformaldehyde concentration varied from 1 to 2% (v/v); and (c) solutions of formol/Ca²⁺ (see above).

Immediately after perfusion, kidneys were removed from the animals and small specimens, which were excised from different levels of cortex and medulla, were placed in the respective fixative for 2-4 h.

Samples from kidneys fixed as in (a) were washed with 0.2 M-sodium cacodylate buffer, and those fixed as in (b) with 0.05 M-sodium phosphate buffer, pH 7.2, containing 15% (w/v) sucrose. They were either dehydrated at this stage and embedded in paraffin wax for light microscopy or were used for immunoelectron microscopy. Samples from kidneys fixed as in (c) were dehydrated for light microscopy.

**Light-microscopic technique by immunofluorescence**

Sections (5 μm thick) were cut from the paraffin-embedded material, and the wax was dissolved with xylene, followed by washing with ethanol, aqueous ethanol solutions and Dulbecco A buffer. The sections were immersed in small volumes of the 10-fold diluted rabbit anti-(hamster Tamm-Horsfall glycoprotein) immunoglobulin G solution (see above) for 10 min at 22°C, and then washed (6 × 2 min) with Dulbecco A buffer. The slices were then immersed in fluorescein-labelled sheep anti-trabbit immunoglobulin G antibodies (8-fold dilution; see above) for 10 min at the same temperature and exhaustively washed (6 × 2 min) with Dulbecco A buffer. The slices were mounted in the same buffer under sealed coverslip.

The preparations were examined with a Leitz microscope fitted with a Leitz dark-ground condenser and a quartz/I2 light source provided with a Turner filter (Gillet and Siebert FITC 3). A barrier filter (Watten B15) was incorporated into the microscope. Alternatively, a Gillet and Siebert microscope fitted with a Zeiss IV FL epifluorescence condenser with an HBO 50 W Hg lamp, and a Zeiss-recommended FITC specific filter set with blue excitation at 450-490 nm was used. Illumination, exposure time and photographic processing were standardized throughout. Photographs were taken on Ilford HP4 and HP6 (ASA 400) films.

**Light-microscopic technique with the use of peroxidase**

Pieces of dehydrated kidney were embedded in paraffin wax and 5 μm sections were cut, mounted and treated as above to remove the wax and finally washed with Dulbecco A buffer. The sections were treated (20 min, 4°C) with 1% (v/v) H₂O₂ in methanol to inactivate endogenous peroxidase (Burns, 1975). The methanol was removed by washing (3 × 5 min at 22°C) with Dulbecco A buffer.

The sections were then treated with undiluted normal sheep serum (20 min, 22°C) to minimize non-specific staining. They were washed after this and subsequent steps with Dulbecco A buffer, unless otherwise stated, and the 10-fold diluted rabbit anti-(hamster Tamm-Horsfall glycoprotein) immunoglobulin G solution was applied (30 min, 22°C) before washing again. Sheep anti-(rabbit immunoglobulin) immunoglobulin G (the 2-fold dilution in Dulbecco A buffer) was next applied followed by washing, before application of the 40-fold diluted PAP reagent.

The sections were thoroughly washed, before staining histochemically for peroxidase activity with 3,3'-diaminobenzidine and H₂O₂ (20 min) by the procedure of Graham & Karnovsky (1966). They were washed with water thoroughly and taken through graded water/ethanol mixtures to absolute ethanol, cleared in xylene, and mounted in XAM resin.

Control sections were prepared in which the anti-(Tamm-Horsfall glycoprotein) immunoglobulin G was either omitted altogether or replaced with normal rabbit serum.

Observations were made and photographs were taken with a Leitz microscope.

**Immunoelectron microscopy**

Small pieces of fixed kidney were placed for 1 h in a mixture consisting of 1 vol. of dimethyl sulphoxide
(Kuhlmann & Miller, 1971) and 9 vol. of either 0.2M-sodium cacodylate buffer, pH 7.4, or 0.05M-sodium phosphate buffer, pH 7.2, depending on the buffer used in fixation (see above). They were then rapidly frozen in liquid N₂ and sections were cut at ~20°C at a thickness of 10-30μm (Avrameas & Bouteille, 1968; Kuhlmann & Miller, 1971). They were washed in Dulbecco A buffer for about 30 min, and then one of the following procedures (I, II or III) was followed.

Procedure I. This was based on that of Avrameas & Ternynck (1971). The slices were immersed for periods of 6-18 h at 4°C in rabbit anti-(Tamm-Horsfall glycoprotein) immunoglobulin G and then washed for 2 h in three changes of Dulbecco A buffer with constant stirring. They were immersed for 6-18 h at 4°C in the Dulbecco A solution of the conjugate composed of sheep anti-(rabbit globulin) immunoglobulin G with horseradish peroxidase, and then washed as before with Dulbecco A buffer before being histochemically stained for peroxidase with 3,3'-diaminobenzidine and H₂O₂ (Graham & Karnovsky, 1966). They were washed with water.

Procedure II. This was based on that of Nakane & Kawaoi (1974). The slices were treated as before with rabbit anti-(Tamm-Horsfall glycoprotein) immunoglobulin G and washed with Dulbecco A buffer before immersion in the Dulbecco A-diluted solution of sheep anti-(rabbit globulin) immunoglobulin G with horseradish peroxidase conjugate formed with the use of NaIO₄ and KBH₄. The slices were then treated as in procedure I.

Procedure III. This was based on that of Sternberger et al. (1970). The kidney sections were immersed for 1 h at 4°C in a 1 in 20 (v/v) dilution of normal sheep serum in Tris/HCl buffer, pH 7.6 (0.046 M in Tris) containing 0.139 M-NaCl, and then without washing in rabbit anti-(Tamm-Horsfall glycoprotein) immunoglobulin G solution for 18 h at 4°C. They were washed in several changes of the Tris/NaCl buffer and then immersed for 18 h at 4°C in a solution of Tris/NaCl buffer containing 1% normal sheep serum of sheep anti-(rabbit globulin) immunoglobulin G. After washing with several changes of the Tris/NaCl buffer, the slices were immersed for 18 h at 4°C in the 40-fold diluted PAP reagent, followed by further washing with the Tris/NaCl buffer. Histochemical staining for peroxidase was carried out as before.

The various slices histochemically stained for peroxidase were washed with water and post-fixed in OsO₄ solution (Palade, 1952) for 1 h before washing again with water. They were dehydrated as above and embedded in Taab's resin (Taab Laboratories, Emmer Green, Reading, Berks., U.K.). Ultra-thin sections (0.05 μm) were cut on a Huxley Ultramicrotome (Cambridge Instrument Co.), mounted on uncoated 200-mesh copper grids, and viewed without further heavy-metal staining at a Miles MR 60C electron microscope at 60 KV.

Results

Studies by immunofluorescence

Immunofluorescent staining of precut sections showed that the immunogen was clearly localized in the ascending limb of the loop of Henle (Plates 1a-1d and 2b) and in the distal convoluted tubule (Plates 1c, 1d, 2a and 2c), with the notable exception of the macula densa (Plate 2a). The glomeruli, proximal convoluted tubules, thin descending limb of Henle's loop and collecting ducts did not fluoresce. Nuclei were not stained.

Several fixation techniques were used and the results in all cases were similar in exhibiting staining of the cells of the ascending limb of the loop of Henle and the distal convoluted tubule. But the most intensely and evenly stained reacting tubules were

### EXPLANATION OF PLATE 1

Fluorescein staining for Tamm–Horsfall glycoprotein

(a) and (b) show thick ascending limbs of Henle's loops in the medulla after formol/Ca⁺² immersion fixation (a) or formol–Ca⁺² perfusion fixation (b). Magnification 240× in both (a) and (b). (c) and (d) show fixed section of kidney cortex with fluorescent distal convoluted tubules and ascending limbs of Henle's loops after formol/Ca⁺² immersion fixation (c; magnification 320×) or fixation by perfusion with formol/Ca⁺² (d; magnification 240×).

### EXPLANATION OF PLATE 2

Fluorescein staining for Tamm–Horsfall glycoprotein

(a) shows staining in the cells of the distal convoluted tubule (DCT), but its absence in the macula densa (MD) cells. Other abbreviations: G, glomerulus. The formol/Ca⁺² immersion fixation method was used. Magnification 360×. (b) shows the thick ascending limbs of Henle's loops in the medulla. Fixation was by perfusion with paraformaldehyde (2% containing glutaraldehyde (0.5%)). Magnification 240×. (c) shows the distal convoluted tubules in the cortex. Fixation was as in (b). Magnification 200×. (d) shows a paraffin section (5μm thick) of renal cortex treated by the light-microscopic immunoperoxidase technique. Fixation was by perfusion with formol/Ca⁺². Cells of the distal convoluted tubules (DCT) are stained, with the exception of the macula densa (MD) region. Other abbreviations: G, glomerulus. Magnification 240×.
obtained with materials fixed by perfusion with formol/Ca²⁺ (Plates 1b and 1d). Immersion fixation with this fixative, although also yielding intensely and evenly stained reactive tubules, was a less valuable procedure because the quality of fixation of the kidney was, in general, not so good. Frequently the tubules collapsed, resulting in a closed lumen (Plates 1a and 1c).

The results obtained after fixation with paraformaldehyde/glutaraldehyde showed greater staining of the luminal border in the medullary ascending limb of Henle's loop than of the remainder of the cell body (Plate 2b). The overall fluorescence was somewhat patchy. On the other hand, staining of sections from the cortex suggested a decreased degree of fluorescence on the luminal border compared with elsewhere in the cell (Plate 2c). In some of the fluorescent tubules from the cortex, small streaks of fluorescent material could be seen to be arising from the basement-membrane side of the cells and passing towards the luminal side (Plate 2c), suggesting staining of the basal plasma membrane with its infoldings.

The macula-densa region of the renal tubule adjacent to the glomerulus was readily identified, because of its slightly taller, but narrower, cells, and consequently with more closely packed nuclei (Plate 2a). Confirmation was obtained by switching to phase-contrast optics. These macula-densa cells, wherever observed, were always negative for the specific staining for Tamm-Horsfall glycoprotein.

Studies with immunoperoxidase at the light-microscopic level.

The examination of 5μm-thick paraffin sections of material fixed in formol/Ca²⁺ or periodate/lysine/paraformaldehyde and stained with the PAP reagent showed a strong apparently cytoplasmic reaction of Tamm–Horsfall glycoprotein in cells of both the ascending limb of the loop of Henle and of the distal convoluted tubule, again with the notable exception of its macula densa (Plate 2d).

Further results were obtained when sections of kidney that had been fixed and treated in the following way were examined. Kidney fixed by perfusion with paraformaldehyde/glutaraldehyde was treated in 30–40μm-thick cryostat sections by procedure II. Tamm–Horsfall glycoprotein was present on the luminal surface of cells of the thick ascending limb of Henle's loop and of the distal convoluted tubule (Plate 3). Once again, the macula densa is free of the glycoprotein.

Another feature, not so clearly evident after the use of immunofluorescence reagents, was the lessened intensity of periluminal Tamm–Horsfall glycoprotein staining in the distal convoluted tubule near the macula densa compared with the other portions.

Studies by immunoelectron microscopy

All the results showed that although Tamm–Horsfall glycoprotein was invariably associated with the cells of the ascending loop of Henle and the distal convoluted tubule, it was always absent in proximal convoluted tubules (Plate 4a), the thin loops of Henle (Plate 4b), the collecting ducts (Plate 5a) and likewise in glomeruli and maculae densae (Plate 5b).

Examination of sections that had been fixed by perfusion with paraformaldehyde/glutaraldehyde and treated by any one of the three procedures used for electron microscopy (see the Experimental section) gave results showing the presence of Tamm–Horsfall glycoprotein on the luminal border of the cells of the ascending limb of the loop of Henle (Plate 4b) and of the distal convoluted tubule (Plates 4a and 5a).

EXPLANATION OF PLATE 3

Araldite section 1μm thick cut from a thicker section of renal cortex that had been treated with immunoperoxidase reagent. The thin section after counterstaining with Azure II was examined by light microscopy. Tamm–Horsfall glycoprotein may be seen intensely on the luminal border of distal convoluted tubule cells (DCT) far removed from the macula densa. The glycoprotein is absent from the luminal surface of the macula-densa cells (MD), but can be seen to be present on the luminal surface of the cells opposite to the macula-densa region. Other abbreviations: PCT, proximal convoluted tubule; G, glomerulus. Fixation was by perfusion with paraformaldehyde/glutaraldehyde. Magnification 550x.

EXPLANATION OF PLATE 4

Identification of Tamm–Horsfall glycoprotein by immunoelectron microscopy methods

(a) shows the presence of the glycoprotein on the luminal surface of cells of the distal convoluted tubule (DCT), and its absence from the surface of those of the proximal convoluted tubule (PCT). Fixation was by perfusion with paraformaldehyde/glutaraldehyde and staining was done by the method of Nakane & Kawai (1974). Magnification 12000x. (b) shows the presence of the glycoprotein on the luminal surface of the thick ascending limb of the loop of Henle (ALH) and its absence on the thin descending limb of Henle's loop (TDLH). Fixation was carried out as in (a). Magnification 10000x.
Tamm–Horsfall glycoprotein was found to occur as far along the distal convoluted tubule as its junction with the collecting duct (Plate 5a). This junction can be recognized by the change in the ultrastructure of the cells, those of the distal convoluted tubule having their mitochondria arranged roughly perpendicular to the basement membrane. On the other hand, the cells of the collecting ducts have their mitochondria randomly distributed and are also extremely vacuolated.

Material that had been fixed by perfusion with the periodate/lysine/paraformaldehyde reagent gave information additional to that already described, namely that there is Tamm–Horsfall glycoprotein not only on the luminal side of the cells of the distal convoluted tubule and of the ascending limb of the loop of Henle, but also on the basal plasma membrane, including its invaginations (Plates 6a and 6b).

Discussion

The results of the present studies performed by the sandwich technique with immunofluorescence show that the Tamm–Horsfall glycoprotein is present in hamster kidney slices only in the ascending limb of the loop of Henle and the distal convoluted tubule. There was generalized fluorescence that cannot be fully interpreted, in part because relatively thick sections were used, and the light microscope has a relatively low resolving power. The glycoprotein was absent in the proximal convoluted tubule, a finding that confirms the results of most other workers. It is possible that some of the previous data suggesting that the glycoprotein occurs within cells of the proximal convoluted tubule of human kidney were due to autofluorescence, which in our experience is quite marked in the hamster, especially with unfixed cryostat sections.

Other workers have also reported the presence of Tamm–Horsfall glycoprotein in the cells of the ascending limb of Henle’s loop and the distal convoluted tubule, but have particularly described staining of the macula-densa region of the nephron (McKenzie & McQueen, 1969; Schenk et al., 1971; Wallace & Nairn, 1971; Hoyer et al., 1974). Our results show that in those sections where macula-densa cells were clearly identified, they did not fluoresce and therefore lack Tamm–Horsfall glycoprotein. A previous report concerning these particular cells in human kidney, in which a claim to the contrary was made by Wallace & Nairn (1971), may require reconsideration, for an examination of the photograph shown in support of their claim does not reveal the morphological characteristics of a macula densa.

Collecting ducts were found to be negative by immunofluorescence, although this part of the nephron has been reported to contain material cross-reacting with uromucoid (Pollak & Arbel, 1969; Masuda et al., 1977). The absence of the glycoprotein in the cells of the collecting duct was confirmed by the results obtained by immunoperoxidase techniques at both the light- and electron-microscopic levels. Indeed, by the last mentioned method, the junction of the distal convoluted tubule with the collecting duct acted as a dividing line between cells producing the glycoprotein and those not doing so (Plate 5a).

The observations made by immunofluorescence were extended by the data obtained by immunoperoxidase methods with the light microscope. In particular, the macula densa of the tubule was shown

EXPLANATION OF PLATE 5

A section of renal cortex showing the junction of the collecting duct and the distal convoluted tubule (a) and a section showing the absence of the Tamm–Horsfall glycoprotein on the luminal surface of the macula-densa (MD) cells and its presence on the other cells of the distal convoluted tubule (DCT) at that point (b).

In (a) Tamm–Horsfall glycoprotein is seen to be present on the luminal surface of the cells of the distal convoluted tubule (DCT) and absent on those of the collecting duct (CD). Other abbreviation: PCT, proximal convoluted tubule. Fixation and staining procedures were carried out as in Plate 4(a). Magnification 6000×. In (b) fixation was by perfusion with paraformaldehyde/glutaraldehyde and the immunoperoxidase technique of Nakane & Kawaoi (1974) was used for staining. Abbreviation: G, glomerulus. Magnification 8000×.

EXPLANATION OF PLATE 6

The presence of Tamm–Horsfall glycoprotein on both the luminal surface and basal plasma membrane, including its invaginations (BI), of cells of the distal convoluted tubule.

Basal lamella (BL) is negative. Fixation was by perfusion with periodate/lysine/paraformaldehyde reagent. In (a) staining was carried out by the method of Nakane & Kawaoi (1974). Magnification 17000×. In (b) staining was carried out by the method of Sternberger et al. (1970). Magnification 12000×.

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to be free of the glycoprotein (Plates 2d and 3). The photograph showing overall cytoplasmic staining of the cells of the distal convoluted tubule (Plate 2d) is of a 5μm-thick paraffin section that was subsequently treated with the reagents. It should be compared with the result depicted in Plate 3, which represents a 1μm-thick section cut from a 30–40μm-thick cryostat section that had been treated by the immunoperoxidase procedure before embedding in Araldite. In the latter type of experiment staining of the Tamm–Horsfall glycoprotein was restricted to the luminal surface of the cells, a result that is related to the method of fixation, or of application of the labelled antibody. It is also noteworthy that the luminal surface of the remaining cells in the transverse section of the nephron cut at the level of its macula densa, like all other cells of the distal convoluted tubule, shows the presence of the glycoprotein (Plate 3).

Further important information was obtained by the studies made with immunoelectron-microscopic methods. Providing periodate/lysine/paraformaldehyde was used as the fixative, the glycoprotein was often seen to be present not only on the luminal surface of the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule, but also on the basal plasma membrane, including the infoldings. Staining of this part of the cell was not seen when other fixatives were used. It seems likely from these results that the plasma membrane of the cells in question is generally associated with the Tamm–Horsfall glycoprotein, rather than just the cell surface exposed to the tubular urine.

Three important features emerge from these studies. Tamm–Horsfall glycoprotein was localized only in the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule as far as its junction with the collecting duct. The glycoprotein is associated with the plasma membrane of the cells in question, both on the luminal surface and on the basal side including its infoldings. The glycoprotein is absent in cells of the macula densa.

From the present observations and those of others concerning the high viscosity of Tamm–Horsfall glycoprotein solutions (Curtain, 1953; Stevenson, 1968; Stevenson et al., 1971), it is tempting to postulate the following role for Tamm–Horsfall glycoprotein in the normal mammalian kidney. The ability of the substance to produce highly viscous solutions suggests that the aggregated molecules of the Tamm–Horsfall glycoprotein can entrap water molecules in a relatively fixed structure. If it is assumed that a similar entrapment of water molecules may also occur in the glycoprotein whilst the latter is still associated with the cell surface, one can envisage a situation in which a barrier of relatively stationary water molecules, occurring within an ordered structure, is present on the surface of the cells of the thick ascending limb of the loop of Henle and of the distal convoluted tubule. This might be expected to lead to a relatively impermeable barrier to the water molecules, but not to the dissolved small solute molecules within the urine in the tubules. A selective passage of molecules across the basal plasma membrane might also be expected to result from the presence of the substance associated with that surface and so prevent loss of water from the particular tubular cells. It is generally accepted that the hyperosmolarity in the medulla of the kidney results from passage of Cl− ions with their accompanying Na+ ions across the single cell layer of the lumen of the thick ascending limb of the loop of Henle, a region of the nephron with relatively high impermeability to water (Roche & Kokke, 1973), and we suggest that Tamm–Horsfall glycoprotein may produce the effect. The presence of the glycoprotein associated with the surface of the cells of the distal convoluted tubule might be expected to decrease the permeability to water of this region, although in the rat at least this is believed to be regulated by vasopressin (Pitts, 1974).

The absence of the glycoprotein on macula-densa cells adds weight to the hypothesis because if these cells function as a sensor for the Cl− or Na+ concentration of the urine at that part of the nephron, as is generally believed (Latta, 1973), it is reasonable to expect their surface to be exposed directly to the urine in the tubules.

The possibility that the Tamm–Horsfall glycoprotein is in some way involved in electrolyte and water transport in the kidney has been suggested before (Lewis et al., 1972; Schwartz et al., 1973), although no specific hypothesis has been put forward.

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